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Description

The present invention refers to equipment and methods for cell selection and, more particularly, to such equipment and methods for trapping individual cells at known locations thereby for use among other ways in selecting at least one sub-population of cells, using defined parameters common to its members, from a more general cell population.

Equipment and methods for selecting and separating sub-populations of biological cells, e.g. those contained in the blood, are known, as is made clear in the prior art portions of claims 1 and 17 herein, see for instance document DE—A—2928790. Several methods which are considered to be important and indicative of the state of the art are briefly described and commented on below.

Separation based on cell adhesion. This method is not very efficient and is not suited for separation of different cells which have the same membranic characteristics, e.g. between cells that stick to glass.

Immunofluorescence separation. This method is based on known binding characteristics of subpopulations of cells to certain antigens and/or antibodies. Their binding to the cells in a later stage can be used to identify the cell. However, this method is quite limited because it cannot be used to separate cells based on measurements of their biological activities or response to the bound matter.

Electrophoresis. This method is based on cell-separation according to the cells' electrical charge. Thus, it cannot be used to separate different groups of cells having the same charge, or mass. Radio assay including radio immunoassay, radio incorporation assay, radio enzymological assay. In this method one cannot separate groups of cells from one another, nor distinguish a subgroup within the group based on its activity or inactivity and response.

Morphology. Distinction between cells is based on their physical appearance. This method is quick but the coarsest of all.

Cell separation according to specific density (gradient technique). In this method cells float upon an isotonic solution of known density, osmolarity and viscosity. This configuration is subjected to acceleration forces by centrifugation at a given temperature and acceleration. The cells, having a specific weight greater than that of the solution sink. Those having the specific density of the solution are suspended in it, and those with a specific density less than that of the solution float above it. The main problem with this method is the cells' compartmentalization within the density gradient, which is influenced by ambient conditions such as temperature, osmolarity, acceleration, e.g. the distance of the interface between the blood and the gradient from spinning axis.

In addition to the above-stated shortcomings of the various prior art methods, a disadvantage

common to all of them is due to the fact that the separated cells nearly always include cells belonging to other than the group or subgroup of interest. Therefore, any diagnosis of the cells, separated by any of these mentioned methods, is necessarily coarse, even if all the procedures have been carried out with the utmost precision.

For example, L. Cereck et al describe a SCM-test (Structuredness of Cytoplasmic Matrix) in Biophys. J., July 1978, Vol. 23, No. 1, p. 395 ff. In said article the authors admit that by the above described gradient method, separated cells contained about 50% of undesired cells, in spite of the great care with which the test was conducted.

Therefore, it is a major object of the invention to provide a method and equipment for selecting a group of cells from other cells and further separate the selected cells from one another. Each of the selected cells, separated from one another, is at a precise known location. All the selected cells are subjectable to common tests, yet the effect on each individual cells is determinable, thereby enabling more accurate diagnosis. The tests and the effects on each cell are performed automatically in order to reduce the testing time, a task performable by relatively unskilled personnel.

The inability to totally separate a particular groups of cells from all other greatly affects the diagnosis accuracy. Furthermore, and most significantly, in the above described methods, cell separation and the following tests conducted thereon are on a macro or batch basis, rather than on a micro basis, i.e. one in which the selected cells are separated from one another and each cell can be separately tested and examined. Any system and method for separating selected cells of interest from other cells and further separate the selected cells from one another, so that each can be separately tested and/or examined, would be of great significance in diagnosing various biological conditions and for other purposes. Testing and examining individual selected cells would eliminate errors, presently existing in many diagnoses, based on inexact statistical criteria.

The invention accordingly provides a method for selecting particular biological cells from other cells to facilitate the observation of at least one selected property of the selected biological cells, which method comprises substantially covering with cells, including the selected cells of interest, the upper surface of a substantially planar carrier of preselected thickness and defining upper and lower surfaces and comprising an ordered array of apertures therethrough, said apertures having a preselected configuration with preselected dimensions at the top and bottom surfaces, and said method including the step of washing the said upper surface of said carrier to remove cells not supported in said apertures whereby only selected cells of preselected dimensions are held substantially in said apertures with substantially one cell per aperture, characterised by providing the top dimension of each aperture larger than its

smallest internal cross-sectional dimension and both the thickness of said carrier and the top dimensions of each aperture of the order of the diameters of the selected cells, and the step of attracting any cell to the aperture in which it is to be supported, or of expelling any cell from the aperture in which it is supported, as a function of a known cell property. It will be appreciated that the method of the invention enables the precise location of a cell in a particular aperture to be identifiable, by the known position of the aperture with respect to coordinates on the x and y axes of said planar carrier.

The invention moreover provides an apparatus for selecting particular biological cells from other cells and observing at least one selected property of the selected biological cells, which apparatus comprises a substantially planar carrier of preselected thickness and defining upper and lower surfaces and comprising an ordered array of apertures therethrough, said apertures having a preselected configuration with preselected dimensions at the top and bottom surfaces, definable as top and bottom dimensions, respectively, whereby when the biological cells are disposed on said top surface, only selected cells of preselected dimensions are held substantially in said apertures with substantially one cell per aperture, characterised in that the top dimension of each aperture is larger than its smallest internal cross-sectional dimension and both the thickness of said carrier and the top dimensions of each aperture are of the order of the diameters of the selected cells, the precise locations of each aperture will be identifiable by its position with respect to coordinates on the x and y axes of said planar carrier.

It will be evident that cells of sizes smaller than those of the selected cells pass through the holes, while much larger cells cannot enter the holes. Once the carrier is rinsed, only selected cells are located in its holes, one cell per hole at a fixed address.

The cells in the carrier holes are then subjectable to biological tests and particular properties thereof are measured on a cell-by-cell basis, to determine which of the cells belong to a particular subgroup, based on their particular properties and their measured parameters. Once a subgroup of cells has been identified, since each cell thereof is at a known address, the addresses of all the subgroup cells are known. Thus, one can subject all the cells to one or more tests, but examine the properties of only each cell in the subgroup by directing the particular measuring and/or diagnosing instruments to the cell's unique address.

Further objects and features of the present invention will become more fully apparent from the following description of several embodiments of the invention based on the accompanying drawings, wherein:

Fig. 1A—1C are schematic illustrations, partly in sectional view, of preferred cell carriers of the invention;

Figs. 2A—2D illustrate one embodiment of a multiple cell carrier holder for carrying out measuring cycles at a plurality of cell carriers;

Figs. 3A and 3B are enlarged sectional views of the holder of FIG. 3;

Fig. 4 schematically exhibits an optical analyzer for scanning individually the cells of the population contained in a cell carrier;

Fig. 5 schematically shows a second embodiment of an optical analyzer;

Figs. 6A—6C exhibit modified holders of the embodiment of Fig. 3;

Figs. 7A and 7B show an embodiment of a flow chamber for the bottom side of an analyzing system;

Fig. 8 is a modified version of the flow chamber of Fig. 7;

Fig. 9 is again another embodiment of a holder and a flow chamber for an advanced analyzing system;

Fig. 10 is a separation unit adapted to receive the holder of Fig. 6 for providing the cell carriers with cells;

Fig. 11 is a sectional view of parts of the embodiment shown in Fig. 10;

Figs. 12A and 12B exhibit a separation unit adapted to receive the holder of Fig. 9 for providing the cell carriers with cells;

Figs. 13A—13C are details of a blood supply element adapted for use with the separation unit of Fig. 12;

Fig. 14 is a sectional view of another embodiment of a blood supply element adapted for use with the separation unit of Fig. 12;

Figs. 15A and 15B show an embodiment of a multi-carrier system for clinical use, wherein the separation and the measuring steps are combined;

Fig. 16 is a schematic overall illustration of an optical diagnosis system according to the invention; and

Fig. 17 illustrates a cell carrier for selectively attracting or releasing desired cells.

The invention will first be described, in a non-limiting way, with regard to selecting and analyzing a particular population of cells of a given type contained in a biological fluid from other populations of cells. In addition, a further selection of a special sub-population can be separated from the particular population selected. More specifically, the invention will first be described in connection with selecting and analyzing a particular sub-population of lymphocytes, which are present in human blood, by first separating the lymphocytes from other types of cells, and then testing the lymphocytes to identify the sub-population or subgroup within the group of lymphocytes.

L. Cercek and B. Cercek in articles published in European Journal of Cancer, vol. 17, 1981, pp. 167—171; same Journal vol. 13, 1977, pp. 903—915; and in Biophysical Journal vol. 23, 1978, pp. 395—405 discuss the excitation and emission-polarization spectra of fluorescein in living cells (the article in the Biophysical Journal)

with relation to the application of the phenomenon of changes in the Structuredness of the Cytoplasmic Matrix (SCM) in the diagnosis of malignant disorders. Briefly, the Cerceks perform the so-called SCM test after first trying to separate a particular subgroup of lymphocytes from other lymphocytes, as well as, other types of cells by the density gradient technique.

This technique as previously pointed out is very unsatisfactory. First, it is very time consuming, as is appreciated by those familiar with the art, and as is clearly apparent from the articles by the Cerceks. Secondly, as the Cerceks acknowledge the finally separated cells do not belong to only the subgroup of interest, but include a large number, on the order of 50% of other lymphocytes. Thus, the analysis of their response to stimulation of the separated cells is very limited. Thirdly, and most significantly all of the stimulations and response measurements, performed by the Cerceks on the separated cells, are done on all the cells in a batch, rather than on a cell-by-cell basis. However, it is clear that a cell-by-cell analysis provides far more information for the understanding of biological implications of the phenomena under study.

The present invention makes it possible to realize such analyses very quickly, and accurately. In this particular case, both speed and accuracy are very important, considering the potential number of cancer diagnosis tests that one may wish to perform. Equally important, the novel invention, both in terms of the system and method, provides capabilities for separating biological cells from one another by placing each separated cell at a known address, to which one can return, for repeated cell observation and/or repeated stimulations followed by subsequent analysis.

Briefly, in accordance with the present invention a large number of cells, e.g. lymphocytes in the blood, which can be thought of as representing a group or population of cells are first separated from all other cells, i.e. from different groups or populations of cells. In the separation process the separated lymphocytes, in addition to being separated from the other cells, are also separated from one another, each being at a known location, hereafter also referred to as an address. All the separated lymphocytes are then subjected simultaneously to selected tests and thereafter each cell is separately investigated to determine whether or not, as a result of the test, or stimulation, it exhibits a particular property. The address of every cell exhibiting said property is recorded. Thus, after all the separated cells have been investigated the addresses of all the cells which exhibited the particular property are known. These cells represent a particular subgroup of lymphocytes within the larger entire group of lymphocytes. Once the cells in the subgroup have been identified, they together with the rest of the lymphocytes may be subjected to one or more additional tests. However, as to investigating the properties of the cells as a result

of these additional test(s) it can be limited to only the cells in the subgroup. Each cell in the subgroup is individually investigated by directing the investigative instrumentation to the cell's unique known location or address. Thus, once the cells in the subgroup have been identified only they are subsequently investigated, while all other cells, though belonging to the same group, but not being part of the subgroup, are ignored in that they are not subjected to any investigation. Consequently once the subgroup has been identified only its cells are investigated, thereby limiting investigation time only to the subgroup cells which are of interest. Also, since the investigation is done on a cell-by-cell basis, more precise data is obtainable for increased diagnosis accuracy. Other advantages of being able to identify cells of a subgroup and investigate each one individually will be discussed hereinafter.

As previously pointed out, in a first step the lymphocytes are separated from the other cells contained in the blood. The separation is performed by means of a perforated cell carrier 1 as shown in Fig. 1A.

The cell carrier 1 may have various configurations of apertures or holes 2, as well as the manner in which they are arranged. In Fig. 1A they are assumed to be arranged in rows and columns along axes X and Y, respectively. The holes are shown as having larger openings at the tops than at the bottoms thereof, as shown in Fig. 1B. In the presently described embodiment the holes are sized to be suited for receiving lymphocytes, among which there are two main sizes of about 7 µm and about 10—15 µm. At the upper surface or side 1t of carrier 1 the apertures have a cross-sectional dimension of approximately 10 µm. The apertures at the bottom surface or side 1b have cross-sectional dimensions of approximately 6 µm. The side walls of the apertures may converge continuously or in steps, as shown in Fig. 1C, towards the opening at the bottom side 1b of the cell carrier.

In general the aperture should be shaped so that either at its bottom side or at a cross-section intermediate sides 1t and 1b the cross-sectional dimension is less than at the top side, so that a desired cell entering an aperture does not pass through the aperture, but rather is held therein. Also it is important to close the carrier thickness between sides 1t and 1b so that the size of the aperture is related to the size of the desired cells so that when a desired cell enters an aperture practically the entire cell is within the aperture, thus preventing it from being washed out during a washing step, as will be described.

The shape of the apertures 2 enables the cells to be effectively held to the carrier by applying means, such as a pressure difference between the upper and the bottom side of the carrier, or electromagnetic forces. Briefly to first separate a particular group of cells from cells of other groups, since the cells in each group are of known size or sizes, which typically differ from those in other groups, the carrier 1 is chosen to have holes

of sizes so that the matter, e.g. blood, containing the various cell groups is placed on the carrier 1, effectively most if not all of the holes are occupied by cells of the group of interest, one cell per hole.

As previously pointed out, the holes 2 in carrier 1 are regularly arranged over or in the carrier, e.g. in rows and columns, to enable a clear identification of the position of every hole 2, for example, by its X and Y coordinates in the plane of the carrier. In the described embodiment the holes are disposed in rows and columns, extending perpendicularly to each other, thereby forming a matrix-like structure. The number of holes is chosen depending on the number of cells to be carried. For example, with 100 holes per row and column there is a total of 10,000 holes to carry 10,000 cells, on the carrier of the described embodiment, each with its unique position in X and Y. The carrier 1 itself may have a circular circumference, as can be seen from Fig. 6C. As shown therein the carrier has a plurality of ears 8, to align the carrier in a holder structure 40 which has a pair of indentations 9 extending from the top recess in which the carrier is supported. A hole extends axially about said recess in holder 40. Other aligning means such as pins or particularly shaped carriers are also within the scope of this invention.

The carrier 1 is made of any appropriate matter, e.g. metals such as copper, gold, nickel, silver or others, or of plastic, which may be provided with electrically conducting portions, extending between the holes 2 as shown in Fig. 17. Thus, the electric potential at any cell-containing hole can be influenced to produce an interaction with the cell's electrical charge. By controlling the potential at various holes the cells therein can be electrically bonded to the carrier as well as be released therefrom.

To practice the method a few drops of the solution containing the lymphocytes, e.g. blood, are dripped onto the cell carrier. The liquid passes through the holes in the carrier. However, the cells remain on the carrier. Since the sizes of the holes 2 are chosen to accommodate lymphocytes only, they enter the holes. Each hole accommodates only one cell. Excessive and other cells are washed off the surface of the carrier, such as cells of sizes so great that they cannot enter any hole, and/or excess cells more than the number of holes. Thereafter, in order to prevent the cells in the holes from leaving the carrier, they may be fixed thereto, by different means, e.g. by covering the carrier by an adhesive, colloidable matter, and by electrically charging it, as well as by electric and/or magnetic fields. Another combined method for isolating said population and simultaneously applying it to the carrier will be discussed later in connection with Figs. 10, 11, 12A and 12B.

Each carrier provided with its group of cells of interest, i.e. with lymphocytes, is placed in a carrier holder of a flow chamber such as holder 10 (Fig. 2D) to provide the necessary environment for the testing or measuring cycles, which will be described later.

In a first embodiment shown in Figs. 2A—2D, 3A

and 3B a plurality of matrices or cell carriers 1 are placed on holder 10 (Figs. 2D and 3A). Only one orientation of the carriers is possible so that the perforations (holes) of the carriers are aligned relative to defined axes, such as X and Y (see Fig. 1A). The holder 10 which is the top of the flow chamber, is removably mounted upon a central part 11 (Fig. 2A) of the flow chamber. The central part 11 defines a plurality of channels 12, each being connected at both ends to one of a plurality of tubes 13 for supplying and discharging a desired solution. The central part is fixed at its bottom by a lower part 14 (Fig. 2B) of the flow chamber comprising a transparent wall 15 which is necessary when using incident and transmitted light techniques for analyzing the cells on the carrier.

As can be seen from Fig. 3A, which is a view on a section perpendicular to the direction of a channel 12 (the solution therefore flowing "into the page"), a flow director 16 ensures that the solution contacts the cell carrier 1. In Fig. 3B a side view of this arrangement is illustrated schematically.

On the holder 10 the carriers 1 of several different individuals (patients) are placed in one row extending along the channels, while a column of carriers of the same person extend perpendicular to the channels. Each channel 12 is related to one type of test so that the number of tests to be run determines the number of channels 12 in the flow chamber.

Any solution, which flows through any one of the channels therefore wets all the cells in the carriers above that channel, each belonging to another patient. The cell carriers 1 may be covered by a glass plate 17 to make possible the use of immersion liquid for the optical scanning system, if necessary.

In Fig. 2A the flow chamber is shown comprising seven channels 12. In such a case cells from each patient are carried on seven carriers, one per channel, while along each channel are supported carriers with cells of different patients, as shown in Fig. 2D. Such an arrangement enables one to stimulate cells of different patients to different stimuli via each channel either simultaneously or successively and then test or analyze the response of each cell to the particular stimulus. Other embodiments to be described also comprise of a plurality of channels. Thus in each multi-channel embodiment the number of cell carrying carriers from each patient is typically equal to the number of channels. However, as will become apparent from the following description the invention is not limited to multichannel arrangements. It was found that cells after being stimulated by certain stimuli and examined can be cleansed and thus returned to their pre-stimulated state to be stimulated subsequently by a different stimulant. Consequently if desired only one cell-supporting carrier per patient can be used. The cells thereon can be successively stimulated and after each stimulation and analysis be rinsed for the next stimulation and analysis steps.

Now, before describing other embodiments of

flow chambers, it is considered desirable to describe particulars of one preferred method and system for individually analyzing the cells placed at defined locations on said carrier and introduced into said flow chamber. To this end reference is made again to the SCM-test as described by L. and B. Cercek et al. in the mentioned publications. According to L. and B. Cercek there are at least two characteristic properties of a subgroup of lymphocytes which are suitable for the SCM test. Acknowledgement of the specific antigen causes a lymphocyte to pass from a rest to a stimulated stage. When fluorescein molecules are imbedded in the lymphocytes, by utilizing a well known phenomenon, called fluorochromasia, the transition from the rest phase to the stimulated phase results in critical changes in the polarization of the fluorescence of the fluorescein in said lymphocytes. The lymphocytes, in which stimulation procedures may evoke such critical changes, differ in at least two characteristic properties from the other lymphocytes; the specific density, and the fact that for these cells a relatively high (control) value of fluorescence polarization is observed only for a very narrow band of the emission spectrum around 510 nm.

This second property is taken advantage of to mark out, or identify the proper lymphocytes among the whole population of lymphocytes and thus avoid the necessity of their physical separation. It is thus the group of lymphocytes which exhibits this particular spectral behaviour on which then all further stimulation effects are examined, while all other lymphocytes will henceforth be neglected by the evaluation technique of the system. Alternately stated, in accordance with the invention, first the carrier is used to separate lymphocytes in a person's drop of blood from other types of cells by means of the sizes of holes in the carrier. The holes are filled substantially by lymphocytes, one cell per hole. Smaller cells passing through the holes and larger cells are washed off the carrier's top surface. Thereafter the lymphocytes on the carrier are rinsed with FDA+PBS, which by fluorochromasia is converted within the cells to fluorescein. Then the fluorescence polarization within a narrow band of the emission spectrum around 510 nm from each cell, is measured and recorded. Only those lymphocyte cells, each of which exhibits a relatively high value of fluorescence polarization, definable as P are regarded as belonging to the particular subgroup of interest. Since the address of each cell on the carrier hole array is known the address of each cell in the subgroup is known. Thus once the cells belonging to the subgroup are known, all subsequent measurements and/or observations which may be performed, are performed only on the cells in the subgroup, whereas all the other lymphocyte cells on the carrier which do not belong to the subgroup may be ignored in that neither measurements nor observations are performed on any of them. The limiting of subsequent measurements or observations to only the

cells in the subgroup greatly reduces analysis time which is of great significance. Furthermore and possibly more important, since the address of each cell is known, the cell's unique response to each stimulant can be recorded to provide unique information, heretofore unattainable due to the fact that measurements and observations were performed on batches of cells or those employing flow systems. Also even when observing a particular cell under a microscope one could not thereafter stimulate it with another stimulant and observe the cell's response thereto. This is due to the fact that heretofore individual cells were not placed in a fixed array with the address of each cell known, so that the measurement and/or observation instrumentation could be directed repeatedly to the same address to observe the same cell.

A suitable criterion may be determined, of the minimum ratio of polarizations measured at two fluorescence emission wavelengths, namely 510 nm and at 515 nm. Therefore, as a first step, the cells of the critical subgroup of lymphocytes are identified by testing said criterion for every single cell on the carrier. Upon transition to a state of stimulation the degree of polarization of the stimulated members of said subgroup decreases to a value of about 0.14 for said emission wavelength of 510 nm. This change of the degree of polarization is examined only for the identified cells of said subgroup.

A system for carrying out these tests for each cell on the carrier will now be described in connection with Fig. 4. The cells on carrier 1 are first typically rinsed with a solution of phosphate-buffered saline (PBS) and fluorescein diacetate (FDA). The latter due to the phenomenon of fluorochromasia is converted within each lymphocyte cell to fluorescein. Then the fluorescein is excited by radiation of wavelength 470 nm upon which it emits its characteristic emission spectrum. The determination of which of the lymphocyte cells on the carrier belong to the subgroup of interest is made by stepwisely scanning each and every cell on the carrier by means of the optical analyzer 20, shown in Fig. 4.

It includes a zirconium lamp (or laser) 21 which serves as a light source peaking at 470.1 nm and 468 nm, thus eliminating the need for an excitation filter to filter any light in the range of interest, i.e. 510 nm and 515 nm. The light is plane polarized perpendicular to the plane of Fig. 4 by a polarizer 22, after passing a focusing lens 21a. The plane polarization is represented by the small circles. The plane polarized light beam strikes a mirror 23 which acts as a beam splitter in that it transmits light of $\lambda > 500$ nm and reflects light below such wavelength. Thus the light from source 21 is reflected to the carrier 1, through a lens 24.

The fluorescence emitted by each cell on the carrier is separately measured and recorded. The fluorescence from a cell passes through mirror 23 and lens 24a to a Glenn-Thompson polarizer 25. Basically, polarizer 25 divides the fluorescence

into two parts: One polarized parallel to the plane of the paper (indicated by the dashes in Fig. 4) which proceeds at the original direction of incidence, and the other polarized normally to the plane of the paper (indicated by the circles in Fig. 4) which is deflected normally to the direction of incidence. Each of the polarized beams is divided into two equal and perpendicular beams by a beam splitter (26, 27). Each of these four newly formed beams passes through an interference filter of 510 nm or 515 nm (28, 29, 28', 29' respectively) and their intensities are measured simultaneously by four photo multiplier tubes (30, 31).

These four measured intensities are stored in a computer system such as that shown in Fig. 16, and the degree of polarization for each wavelength, i.e. $\lambda=510$ nm and $\lambda=515$ nm is calculated. The degree of polarization is defined as

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

After calculation of P_{510} and P_{515} their ratio, i.e. P_{510}/P_{515} representing the control value is calculated in real time. The address of each cell in terms of its X and Y coordinates are known and is stored together with its control value. After all the cells have been examined and their control values determined and stored it is very simple to determine the cells having a control value of not less than 1.3. It is these cells that belong to the subgroup of interest. Once this determination is made all subsequent measurements or observations of the response of the cells to various stimulating agents are performed on the cells in the subgroup only and all other cells are ignored. For example only the cells in the subgroup are reexamined to determine which of them exhibit a change in the degree of polarization sufficient to identify them as active and thus capable of identifying a particular antigen.

It should be apparent that to test each cell individually the optical analyzer 20 (Fig. 4) has to have an optical resolution in the range of one cell diameter which is achievable with a microscope objective. The carrier with the cells is stepwise displaced under the microscope from one perforation to the neighboring one. A precise mechanical displacing system, as described in Fig. 16 is thus necessary.

In another embodiment of the optical analyzer, the need to stepwise displace the cell carrier is avoided by using a laser as excitation light source. In Fig. 5 this embodiment is schematically illustrated. A laser beam 131 of appropriate wavelength passes through a controlled deflecting optical element such as, e.g., a rotating mirror 130. The laser beam 131 has a cross-section which corresponds substantially to the size of a cell. By means of the deflecting element 130 the beam 131 scans the cells in the holes sequentially, thereby exciting each cell, one after the other. At any given time only one cell is hit by the laser beam and therefore only this cell emits fluorescence light at that moment. The optical analyzer 131X, disposed on the other side of the carrier 1

has a visual field, covering the whole surface of the carrier 1. The moment of the receipt of an emission signal is the intensity of this signal correlated with the position of the scanning laser beam 131, hence each received and analyzed light signal is correlated with the position of the respective cell from which it has been emitted. As can be seen from Fig. 5 the excitation is made from the large side of the holes 2 in the carrier 1. For the optical analysis, on the other hand, emission light leaving each hole through the narrow end is preferably used for reasons which will be explained below.

Having explained preferred analyzing systems using the invention, it should be well understood that analogous systems for measuring other parameters may be used, provided that focusing on each single cell on the carrier is possible. Examples of measurable parameters include light intensity, optical density, index of refraction, electromagnetic properties, absorption and scattering. Furthermore, the scanning procedure is not limited to beams such as visible light, U.V., I.R. and electron optical systems, but may also include probing via physical contact at each cell. Other examples of measurable or observable properties include nuclear magnetic resonance (NMR), pH value as well as cell morphology and changes thereof in response to different stimulants. For example, one can direct the output of a microscope pointed at any cell to a pattern recorder to produce a two-dimensional record of the cell's pattern. Cell temperature measurements and/or temperature changes may be performed and recorded. In summary, any one or more measureable or observable property of a cell may be performed on a cell by cell basis. Since the address of each cell is known one can always return to the same cell for additional measurements and/or observations. All measurements and observations for each cell can be recorded to obtain unique information for each individual cell. This information can be correlated to provide insight and diagnosis, heretofore unattainable.

An embodiment of the invention for practical clinical use will now be explained in connection with Figs. 6A—6C which show a modified holder 40 for a plurality of cell carriers 1. The holder 40 is wave-formed to enable its troughs 41 to be immersed in the solutions flowing through the channels 12 at a higher level. The cell carriers which are mounted on the bottom of the troughs 41, can be wetted to rinse or otherwise stimulate the cells both from the upper and the bottom sides. Therefore, in this embodiment there is no need for flow directors, as previously explained in connection with Figs. 2A, 3B. As has been described in connection with Figs. 2A—2D, the cell carriers positioned on the same trough 41 belong to different patients. In spite of this there is no danger of any mixed lymphocyte stimulation effect because there is no physical connection between carriers. Even if a cell would disconnect from one carrier, the chances of it being rinsed

out are much higher than that of it being deposited on another carrier. In Fig. 6A carriers are shown only in one trough. However in practice for each patient a carrier is present in each trough.

In Fig. 6C the carrier 1 is shown as being removable from holder 40. However to define its hole array in X and Y, it includes ears 8 locatable in indentations 9.

Since the cell carriers 1 of the present embodiment are immersed in the solution flowing through each channel 12, the microscope of the optical system for cell scanning is provided with a quartz sleeve 42 (Fig. 6B) dressed on its objective cylinder. The channels 12 and the troughs 41 have dimensions which enable the relative movements of the objective and the carriers necessary for scanning the whole surface of each of the carriers.

As indicated above, to select the subgroup of cells based on the above described control value the channels are first supplied with a PBS+FDA solution during the control measuring cycle for identifying the proper cells on each carrier belonging to the subgroup. Thereafter, for determining the reaction of the selected cells to different stimulating agents each channel is supplied by a different stimulating agent, e.g. phytohemagglutinin (PHA), EF, CaBP, tumor extracts, or any other desired mitogen or antigen. Then the responses of only the selected cells are examined and recorded.

For the above stimulators it was discovered that stimulation of cells by one stimulator, does not affect any following stimulation if the stimulator is rinsed and/or neutralized before the next stimulation test, in order to prevent any direct interaction or any competitive effect between them. Furthermore, it has been found that bonding the cells to the carrier has no effect on their activation. As a consequence, the stimulation procedures can be repeated on the same cell at the same location on the carrier, and this with different activating agents. Thus, an exact profile of the response of each individual cell of the subgroup to activation can be received as a function of time and it is therefore now possible to know the exact number and response of the activated cells and their places on the matrix which remains the same during and after the above described measuring cycles.

Most of the carrier holder systems, described above, were designed for top scanning, i.e., for analyzing the emitted fluorescence light from the large upper side of the holes in the carrier, which allows the use of the same optical system for optical examination and analysis of the cells. In alternative embodiments, which will be described herebelow, the optical analyzer is placed to receive the emission light passing through the narrow side or bottom of the holes 2. Thus, disturbing effects, caused by light emission of fluorescein, which leaks out of the cells and is present in their surroundings can be eliminated. The light emitted by the surrounding fluorescein represents an undesired optical background.

Looking at the cells from the narrow sides or bottoms of the holes permits the reduction of this background substantially, since the narrowing conus acts as a shield against undesired emission light. Moreover, in the case of the excitation light entering the holes through their large sides, or tops, reflections at the conical walls may occur, whereby incident light as well as fluorescence light is reflected back. Another advantageous effect, caused by carrying out the optical analysis in the mentioned way is that at every location on the carrier only the emission light of the cell trapped within the respective hole is received, whereas other cells which may in exceptional cases be present at the upper surface of the carrier do not influence the measuring results. Still another advantage resides in the fact that due to the smaller size of the openings it is much easier in practice to analyze the emission light of each cell separately, without the danger of cross-talking between adjacent cells if the adjusting mechanism of the optical system relative to the holes is not of extreme precision.

By means of the Figs. 7A, 7B, 8 and 9, various embodiments are illustrated which enable the optical analysis to be carried out as explained above. In a first embodiment for use with a microscope optical analyzer (Figs. 7A and 7B) the bottom wall 110 of the flow chamber comprises elastic (rubber) glass holders 111, each carrying a glass plate 112 adjusted relative to an above located cell carrier 1. The elastic glass holder 111 provides a fluid-tight seal between the glass plate 112 and the bottom wall 110 of the flow chamber and enables the objective 113 of a microscope to be moved close enough to the cell carrier 1 for scanning its individual locations or holes from below (Fig. 7B). If the objective 113 of the microscope is in its lower position, the channel of the flow chamber then is opened to its initial width. In a modification (Fig. 8) of this embodiment, the bottom and side walls of the flow chamber are integrally made of rubber.

In a second embodiment (Fig. 9) the optical analysis is made from the upper side. However, the holder 114 for the cell carriers 1 is placed upside-down on a bottom portion 115 of the flow chamber, after being provided with cells in a special unit (which will be described in connection with Figs. 12A and 12B, such that the conical holes in the carriers 1 flare downwardly. In order to hold the cells in place and to effectively bond them to the carrier a pressure difference is applied between the bottom portion 115 (fig. 9) and an upper portion 116 of the flow chamber, the fluid in the bottom portion 115 having a slightly higher pressure than in the upper portion 116. Sealing ledges 117 prevent the two portions of the flow chamber from leaking. Using this embodiment the optical analyzer of Fig. 4 can be used for scanning the cells on carriers 1 without giving up the above-described advantages.

Returning now to the problem of providing the cell carriers with cells of a certain desired population or group, which in principle could be done in

substantially conventional manner as described before, Figs. 6, 7A and 7B illustrate a system for simultaneously separating said cell population from other groups of cells other than by the conventional disadvantageous methods of cell separation. The present embodiment is also described with regard to the separation of lymphocytes from the other blood cells, for use in the above described SCM-tests.

As can be seen from Fig. 10 and Fig. 11 the holder 50, which is insertable onto the flow chamber of Fig. 6A rests on an arrangement of pipes 51, each being subdivided lengthwise in an upper part 53 and a lower part 52. The lower part 52 forms a fluid (air or liquid) conduit of lower pressure to drain liquids and improper blood cells from the upper part 53. The upper part comprises bridges 54, under which there are drainage holes 55, and between which there are suction holes 56, aligned with the carriers 1 on the holder 50. In Fig. 10 carriers for supporting cells from only one patient are shown. The upper and the lower parts 53 and 52 are supplied by a fluid, say a PBS-solution. As becomes clear from the sectional view of Fig. 11, the fluid in the upper part passes under the bridges 54 and through appropriate slots 57 in the holder 50. The fluid in the lower part 52 is forced by projections 58 to flow with a higher speed in the region of the draining holes 55 and the suction holes 56, thereby creating a local subpressure in these holes. Therefore the fluid initially flowing through the upper part 53 is partly drawn to the lower part through said holes.

A blood supply element 60, removably placed upon the holder 50, is provided for supplying the carriers 1 with blood. Legs 61 of supply element 60 prevent fluid from passing from one row of slots 57 in the holder 50 to another.

As a theoretical basis for understanding the cell separation by the above unit, the following facts are emphasized:

- a) the size of the responding lymphocytes is $\sim 7 \mu$.
- b) the size of macrophages, granulocytes is $\sim 20 \mu - 35 \mu$;
- c) the size of erythrocytes can reach $3 \mu - 5 \mu$;
- d) there are large lymphocytes— 15μ ;
- e) the size of the platelets—negligible;
- f) cells can burst when left in distilled water;
- g) the life span of an erythrocyte in distilled water is much less than that of a lymphocyte.

The cell carrier holder 50 is first placed on the pipe arrangement, such that the carriers of the first row (normal to the channels) are placed above the holes. The supply element 60 is placed with its legs 61 being on either side of the cell carriers. The whole system is assembled, as shown in Fig. 11. A syringe with full blood from a patient is placed in a syringe holder 62. In Fig. 11 two such holders are shown for two different patients. The blood flow in each of the pipes is controlled by applying suitable pressure on the syringe. Blood arrives at all the exits of pipes P1 and P2 (from 2 different patients) after the first few pressure pulses.

At a certain stage a pressure pulse will cause a drop of blood to fall on each cell carrier. The size of the holes in the carrier will not allow the blood to pass from one side of the cell carrier to the other. To this end a sub-pressure is formed in the lower half of the separation pipe, as described above, by running PBS through this part of the pipe. The blood is sucked immediately under the carrier.

5 The smaller cells will pass through the carrier and will be rinsed away with the PBS flow. Those with a size similar to that of the top of the holes of the carrier, e.g. 7μ , will stop on the carrier and the biggest will rest above carrier. In order to prevent blocking of the carrier, the blood supply is stopped and PBS flows across the upper part of the matrix for washing away the bigger cells. Most of them are sucked into the drainage holes 55 (Fig. 11). The minority of the cells get to the next carrier (in the direction of the stream) and pass out. As previously pointed out, all cell carriers, placed perpendicularly to the extension of the channels are filled with the blood of one donor. Therefore there is no problem of blood being mixed from different donors.

10 In a next stage the upper flow is stopped and another drop of blood is dripped and the cycle is repeated as often as necessary. After a few drops of blood a so-called "upper bursting wash" is carried out. The process is continued until the carrier is sufficiently filled. A rough test of this can be made by testing the electrical resistivity of the carrier after each drop. Distilled water flows for any desired time and causes the erythrocytes to burst. The distilled water causes cells to swell, and therefore, the erythrocytes burst, while the lymphocytes strengthen their hold in the carrier holes. At the end of the desired time interval PBS is introduced. The substances set free from the bursted erythrocytes cannot influence the lymphocytes since there is a permanent flow of solution washing these substances away electrically charging or recharging the matrix, or applying or terminating electromagnetic fields is analogous to vibrating the matrix via ultrasonic or other techniques which can also be used. This procedure can be added and correlated with the stages of washing. Less than 1 cc. of blood will be necessary from each patient P₁, P₂, etc.

15 This separation process lasts about 5 minutes at the most. There is no limit to the number of blood samples from which cells can be simultaneously separated. The holder 50 is then removed from the separating system and inserted into the flow chamber of Fig. 4 for the optical scanning operation as described above.

20 A similar separating system but adapted to the holder 114 of Fig. 9 will be explained by means of Figs. 12A and 12B. A base plate 120 is provided with channels 121 for fluid flow, causing the necessary local subpressure in the region beneath the carriers 1 and draining holes 55. To this end projections 122 are formed on the base of the channels 121. The holder 114 is removably placed on the base plate 120 so that its carriers 1

are aligned with the projections 122 as can be seen from Fig. 12B, such that their conical holes open upwardly, i.e. towards an overlying removable supply element 123 which is similar in function to the supply element 60 of Fig. 10. The supply element 123 may supply the cell carriers with cells merely by the action of pressure as has been explained in connection with Fig. 10. It is, however, possible to enhance the efficiency of blood supply by providing smearing elements which are displaceable with respect to the carriers, as illustrated in the Figs. 13A, 13B, 13C and 14. In Figs. 13A, 13B and 13C an embodiment is shown, having slide plates 124 extending in the supply element 123 aligned with the channels 121.

At each outlet of a supply conduit a resilient smearing element 125 is arranged as can be seen from Figs. 13B and 13C. In Fig. 13B a cross-section of the smearing element 125, perpendicular to the direction of a slide plate 124 is shown, whereas Fig. 13C illustrates a cross-section along the extension of said plate 124. The width of the resilient smearing element 125 substantially correspond to the side length of a carrier and it forms a small outlet for linearly sweeping over the carrier surface, when moving the slide plate 124, such that each carrier is coated by a thin layer of cells. Thereafter the above described washing steps are performed.

In Fig. 14 another embodiment of the smearing element is shown in a cross-section, perpendicular to a channel 121. In a swivelling bar 126 extending along each channel 121 a blood conduit 127 is formed, which at each carrier 1, is provided with an outlet, having a distributing brush 128. When supplying blood to the carrier, the swiveling bar 126 is swivelled several times, thereby brushing the cells onto the carrier 1.

While in the above embodiment blood supply and "rough" separation is performed by means of a special separation unit whereafter the holders 40, 50 and 114, respectively, have to be placed on a flow chamber for optical scanning, in some cases it may be desirable to eliminate this step. In a further embodiment of the invention which is shown in Figs. 15A and 15B the cell separation and the optical scanning operation are therefore combined in one apparatus.

A supporting system 70 is provided with surface channels 71, 72 extending transversely to each other and inner conduits 73, 74 also extending transversely to each other. At every junction a carrier 1 is arranged on a rotatable holder 75, a section of which is shown in Fig. 15B. At its base a pinion 76 is formed which cooperates with a respective rack 77, extending through the supporting member 70. One rack 77 drives all the holders 75 of the respective column. Linear movement of this rack 77 causes rotation of the holders 75. The direction of introducing blood for rough separation, i.e., for separating the group of lymphocytes from the other group of blood cells, is perpendicular to the plane in which the cell carrier is scanned under the microscope. Thus, after

separation of the lymphocytes from other blood cells the holders 75 are rotated 90° for the scanning operation.

In order to make possible the technique of "transmitted light" (measuring light exiting the bottom end of a hole) in the above embodiment, the portion of the channel which crosses the holder 75 under the cell carriers is a pipe of glass 78 which is divided lengthwise. This pipe is arranged so that its open side is directed towards the carrier (see Fig. 15B). In this way horizontal liquid flow through the holder is made possible, while at the same time light is transmitted in a vertical direction. The subpressure in this system is caused by making the inner conduits 73, 74 closed and thinner, while the upper channels 71, 72 are wider and open. The same effect can be achieved by other techniques, such as increasing the flow rate in the inner conduits with respect to that of the upper channels.

The procedure can be summarized as follows: With the aid of a 0—90° controller the position of the holders 75 is determined. In a first stage, when the "rough separation" is carried out, the channels 71 and conduits 73 are in operation. Upon completion of this stage, the holders 75 are rotated by 90°. Thus the channels 71 and conduits 73 are blocked or closed and the channels 72 and conduits 74 are opened.

In this embodiment a blood drip-head may be attached to the scanning head, e.g. microscope. Then in response to command signals from a controller, e.g. a computer the separation and the optical scanning are performed automatically and without need for a trained operator. The operator need only place the syringes, as shown in Fig. 6 and to change the holders 75, after completion of the tests.

In Fig. 16 to which attention is directed, an overall system of cell separation, scanning and analysis (diagnosis) is shown. A flow chamber 81, as described above, is mounted on a table 82 which is displaceable in three axes X, Y, Z by respective computer controlled step motors 83, 84, 85. The optical system includes a microscope 86 with an optical analyzer 87, as described in Fig. 4. An excitation light source 88, e.g. a zirconium lamp, uses the same optical system in reverse direction. In a solution tank 89 all the solutions necessary for cell separation and testing are stored. By a solution control unit 90 the supply of the respective solution is controlled. In order to stabilize the fluorescein concentration in the cells, which may influence the absolute polarization values, an electro-optical mechanical feedback control is used, wherein the intensity of the fluorescence emission light is periodically measured and compared with a reference value. Any deviation of the measured value from the reference value may be used to cause a change in the concentration of FDA in the PBS solution. The analysis of the measured value may be carried out by any well known computer system. A precomputer interface 91 serves to transform the measured values into computer-readable infor-

mation which is typically digital. In a computer 92 the necessary calculations and identification steps are performed and stored in a memory 93. A post computer controller 94 generates the control signals for the step motors and the solution control unit.

The operation of the above system may be summarized as follows: After the rough separation procedure, the flow chamber is fixed on the table 82. The microscope is adjusted. Henceforth the test proceeds automatically. A PBS+FDA solution is introduced through the channels and conduits. Part of it penetrates through the carriers from the upper channels to the lower conduits and part of it continues to flow through the upper channels, washing the cells from above. After a chosen pause, e.g. 20 minutes, the scan begins. The polarization of every single cell is measured at the desired wavelengths. There is no danger of over-exposure of the cell to the exciting light, e.g. 470 nm, because scanning is performed very rapidly.

The optical information—after conversion into an electric current pulse—is fed into the computer, evaluated and stored in the memory. Every single cell is identified in the memory according to its coordinates, i.e. address on the carrier. From this stage on, everything that can be learned about each single cell will be stored in the computer relating to its address.

The collection of data may be summarized as follows: The control values of cells of all patients whose carriers are aligned in one channel will first be determined. Then the scanning head will be transferred to the next channel (by lowering the table and moving it aside) and will be used in the determination of the control values of all the cells on the carriers aligned in the second channel. Simultaneously with the data collection from the second channel a stimulating solution will be introduced into the first channel. Upon termination of the data collection from the second channel the scanning head is transferred to the third channel and a second stimulating solution is introduced into the second channel etc.

After data collection from all the carriers the scanning head will be returned to its first position. Then the scanning operation is repeated on the stimulated cells. This time the data collection will be selective and only cells which meet the described optical criterion, i.e. those belonging to the particular subgroup, will be reread. Therefore, the information which will be accumulated in the computer will be cell position, control values, values of polarization after stimulation with PHA, values of polarization after stimulation with CaBP, SCM-response ratio, (See L. Cersek et al. in *Europ. J. Cancer*, Vol. 17, pp. 167—171, 1981), polarization values after stimulation with specific tumor stimulators, and the like.

The distinction between the cell carriers of different patients may be made by magnetic or optical coding which can be fixed on the holders during the rough separation stage. A magnetic or optic reader can be attached to the optical scan-

ning head which will read the patient's code and transfer it to the computer. All the information pertaining to each patient may be transferred to a predetermined place in the computer memory.

By this system the exact number of activated lymphocytes can be determined for every stimulating agent. To one familiar with the art, the present invention permits cancer diagnosis at a very early stage. Although the present invention has been described primarily in conjunction with cancer diagnosis it is obvious that the inventive method and system are not limited thereto. Generically they provide a method and means for rapidly conducting biological assays leading to new clinical diagnosis and treatment as well as to new applications in the field of biotechnology and bioengineering.

As mentioned above, the exact position of each activated cell on a carrier is determined and stored. Therefore it is possible to isolate a desired group or subgroup of cells on the carrier by selectively removing all other cells from the carrier, so that only the subgroup of cells remains thereon, or by releasing and removing only the cells of the subgroup from the carrier. To this end use may be made of the known fact that cells are not electrically neutral but possess electrical surface charges. This fact may be used in the above described embodiments for bonding or otherwise securing the cells to the carrier. The same effect may be used to selectively release or hold desired cells. This may be achieved by a modified embodiment of the cell carrier of Fig. 1A, which will be now be explained in connection with Fig. 17. The outer shape of the carrier 100 is the same as shown in Fig. 1A. However, carrier 10 is provided with electrical conductors 101 extending between the holes 102 in grid-like configuration and being electrically isolated from each other. At the periphery of the carrier the conductors are connected in a known manner (IC-technique) to a computer controlled switching arrangement for selectively influencing the electrical potential of every conductor 101. For securing the cells to the carrier all conductors 101 may be held at the same potential opposite the cells' charge potential, resulting in electrical attraction of the cells. For releasing any cell, say the one in the hole marked A in Fig. 17, the neighboring conductors V_{x2} , V_{x3} , V_{y1} , V_{y2} may be set to an appropriate potential to cause the ejection of the cell in hole A from the carrier.

The cell carrier 100 may be produced by a multilayer technique, known from IC-production. In case an ionic solution, such as PBS, is used, measures should be taken to avoid undesired influences of possible surface charges on the holder. For this purpose it may be isolatedly coated and provided with conducting elements ending in the channel. This would cause ions to be attracted and neutralized, thus preventing the formation of an ion cover over the holder surface which may affect the potential of the carrier. Another possibility is to use non-ionic, organic solutions such as lipids for flowing the carrier in this stage of the procedure.

The separation of particular cells in accordance with the invention from all other cells is uniquely applicable in the field of clinical treatment in the production of clones. Clones may be produced from particular cells which were selected from other cells in accordance with the present invention based on any chosen property. For example, it is well known that the body of a person, afflicted with certain diseases, e.g. skin cancer, produces identifiable cells to combat or kill the disease. However to be successful, a large number of such cells, hereafter referred to as killer cells, may have to be present in the body. With the present invention, blood, lymph nodes and different body tissue, containing some killer cells, may be used as the source of such cells. After separating them, as heretofore described, from all other cells, the killer cells may be multiplied by appropriate cell growing techniques, and then introduced into the patient, from which the original cells were received to fight the disease. In such a case, no cell rejection is expected since the cells originated from the patient's body. Thus, the present invention can be used to provide a person's body with enough cells to fight its own affliction.

It should be pointed out that whereas heretofore the separation between cells of interest on the carrier and other cells can be accomplished by expelling or removing either the cells of interest from the carrier so that only the other cells remain on it, or by removing the cells which are not of interest and leaving only the selected cells on the carrier, if desired one can produce such separation by destroying such as by killing the cells which are not of interest while they are in apertures of the carrier so that the only live cells remaining on the carrier are the cells of interest. The killing of cells in the apertures, i.e. in-situ may be achieved by directing a laser beam to each cell at its known address as well as by similar or analogous means. A killed cell, i.e. a dead cell even though on the carrier can thus be regarded as being separated or removed therefrom since for all practical purposes, once killed it is disregarded. As used herein the term "expelling" of a cell is intended to include removing a cell from the carrier or killing it in-situ.

As to multiplying cells of interest it should thus be apparent that it can be done after:

a) the cells to be multiplied were removed from the carrier bearing live cells which are not of interest on it;

b) removing the cells which are not of interest from the carrier and thereafter multiplying the cells of interest for growth purposes; and

c) killing the cells which are not of interest when they are still in the apertures and multiplying the cells of interest in-situ, i.e. while they are in their apertures of the carrier.

While the principles of the invention have been described in connection with specific systems, applications and methods, it is to be understood that this description is provided for explanatory purposes only and is not intended as a limitation of the scope of the invention.

Many new applications in biological research, clinical treatment and industrial production are opened by the present invention. It is expected, and has been established to a satisfactory extent, that there are optical parameters related to the cyclic phase of the cells. By this invention it is possible to differentiate a cell population according to the cells' age, their cycle stage and their inherent function, and to conduct respective examinations. A clinical application of the above resides in early detection of leukemia which is characterized by the presence of a high number of young cells of a certain type or types in the blood, and in the bone marrow.

As another clinical application immunoreactivity tests for organ transplantations may be performed. To this end a preparation of the transplant is used as stimulating agent in the invention.

A general and major feature and advantage of the invention is the fact that the time required for biological experiments and tests is substantially shortened since cell identification and testing is carried out in a substantially shorter time than in conventional biological methods wherein natural developments often have to be reproduced under artificial conditions, leading to uncertain results, necessitating extensive statistical evaluations. The invention reduces the influences of the surroundings allowing numerical analysis with a minimum of statistics. The time requirement to perform measurements with the present invention is very short in absolute terms as compared to the prior art, thereby reducing the effect of changes in the environmental conditions of the surroundings, such as temperature, humidity, etc.

Although particular embodiments of the invention have been described as illustrated herein, it is recognized that modifications and variations may readily occur to those skilled in the art and consequently, it is intended that the claims be interpreted to cover such modifications and equivalents.

45 Claims

1. Apparatus for selecting particular biological cells from other cells and observing at least one selected property of the selected biological cells, which apparatus comprises a substantially planar carrier (1) of preselected thickness and defining upper and lower surfaces and comprising an ordered array of apertures (2) therethrough, said apertures having a preselected configuration with preselected dimensions at the top and bottom surfaces, definable as top and bottom dimensions, respectively, whereby when the biological cells are disposed on said top surface, only selected cells of preselected dimensions are held substantially in said apertures with substantially one cell per aperture, characterised in that, the top dimension of each aperture is larger than its smallest internal cross-sectional dimension and both the thickness of said carrier and the top dimensions of each aperture of the order of the

diameters of the selected cells.

2. Apparatus according to claim 1 wherein the top and bottom dimensions of each aperture are in the range of micrometers.

3. Apparatus according to claim 1 wherein the apertures are dimensioned to hold lymphocytes, one per aperture.

4. Apparatus according to any of claims 1 to 3, in which the precise location of each aperture is identifiable by its position with respect to coordinates on the x and y axes of said planar carrier.

5. Apparatus according to any of the preceding claims, which further includes first structure means (10;4 0) for supporting said carrier at a defined location thereon, whereby each aperture of said carrier is located at a known location, definable as an address, with respect to said structure means, instrument means (Figures 4, 5, 6B) for observing and/or measuring at least one cell property, and control means (94) for controlling the relative movement and operation of said instrument means with respect to said carrier, and thereby enabling the observation and/or measurement of at least one cell property of a cell in any particular one of said apertures, based on its address, to be effected.

6. Apparatus according to claim 5 wherein said instrument means comprises optical scanning means (86, 87) for determining optical properties of any of said cells.

7. Apparatus according to claim 5 wherein said first structure means (10) includes means for supporting a plurality of spaced-apart carriers, each carrier having cells in its apertures, and second structure means (11) selectively positionable relative to said first structure means, for enabling the cells on each of said spaced-apart carriers to be simultaneously stimulated by pre-selected matter, with said control means (94) being adapted to direct the relative movement of said instrument means with respect to any one of said carriers, and thereby enabling the observation and/or measurement of at least one cell property of a cell in any particular one of said apertures, based on its address, to be effected.

8. Apparatus according to claim 7 wherein said first and second structure means are positionable with respect to one another, whereby said cells are stimulatable by being wetted through either the top or bottom end of said apertures.

9. Apparatus according to claim 7 wherein said second structure means includes means for containing different stimulating matters therein, whereby cells in at least some of the apertures of said spaced-apart carriers may be stimulated simultaneously by said different stimulating matters.

10. Apparatus according to any of claims 1 to 6, and including also force producing means for attracting or expelling any of said cells to or from the apertures.

11. Apparatus according to claim 10 wherein said force producing means comprises means arranged in a predetermined pattern with respect to said apertures for attracting or expelling one or

more of said cells to or from the apertures by electromagnetic means (101).

12. Apparatus according to claim 10 wherein said force producing means comprises means (Figure 11) for producing a pressure difference across at least a selected portion of said carrier(s).

13. Apparatus according to claim 10 wherein said force producing means comprises means (115, 116, 117) for enhancing the adherence of the cells in said apertures.

14. Apparatus according to any of claims 10 to 13, wherein said first structure means (10) includes means for supporting a plurality of spaced-apart carriers, each carrier having cells in its apertures, and second structure means (11) selectively positionable relative to said first structure means, for enabling the cells on each of said spaced-apart carriers to be simultaneously stimulated by preselected matter, with said control means (94) being adapted to direct the relative movement of said instrument means with respect to any one of said carriers, and thereby enabling the observation and/or measurement of at least one cell property of a cell in any particular one of said apertures, based on its address, to be effected.

15. Apparatus according to claim 14 wherein said first and second structure means are positionable with respect to one another, whereby said cells are stimulatable by being wetted through either the top or bottom end of said apertures.

16. Apparatus according to claim 14 wherein said second structure means includes means for containing different stimulating matters therein, whereby cells in at least some of the apertures of said spaced-apart carriers may be stimulated simultaneously by said different stimulating matters.

17. A method for selecting particular biological cells from other cells to facilitate the observation of at least one selected property of the selected biological cells, which method comprises substantially covering with cells, including the selected cells of interest, the upper surface of a substantially planar carrier of preselected thickness and defining upper and lower surfaces and comprising an ordered array of apertures therethrough, said apertures having a preselected configuration with preselected dimensions at the top and bottom surfaces, and said method including the step of washing the said upper surface of said carrier to remove cells not supported in said apertures whereby only selected cells of preselected dimensions are held substantially in said apertures with substantially one cell per aperture characterised by providing the top dimension of each aperture larger than its smallest internal cross-sectional dimension and both the thickness of said carrier and the top dimensions of each aperture of the order of the diameters of the selected cells, and the step of attracting any cell to the aperture in which it is to be supported, or of expelling any cell from the aperture in which it is supported, as a function of a known cell property.

18. A method according to claim 17 which further includes the step of treating the cells in the carrier apertures following a prior stimulation substantially to return them to their prior state.

19. A method according to claim 18 which further includes the steps of neutralizing the cells in the carrier apertures following a prior stimulation and then stimulating the cells with different matter.

20. A method according to any of claims 17 to 19 which includes the step of multiplying selected cells on the carrier in order to increase their number.

21. A method according to claim 17 which includes the steps of expelling selected cells from carrier apertures and subsequently multiplying them in order to increase their number.

22. A method according to claim 17 which further includes the step of killing at least one cell which is not among the selected cells while the cell is in situ, namely in an aperture of the carrier.

23. A method according to claim 22 which further includes the step of multiplying the live cells on the carrier in situ.

24. A method according to claim 23 which further includes the step of observing the multiplication process of at least one cell which multiplies in situ.

25. A method according to any of claims 17 to 24, wherein the precise location of each aperture is identifiable by its position with respect to coordinates on the x and y axes of said planar carrier.

26. A method according to any of claims 17 to 25, wherein said attracting and/or expelling step is effected by electromagnetic means.

27. A method according to any of claims 17 to 25, wherein said attracting and/or expelling step is effected by means for producing a pressure difference across at least a selected portion of said carrier.

28. A method according to any of claims 17 to 25, wherein the adherence of cells in said apertures is enhanced by means adapted for this purpose.

Patentansprüche

1. Vorrichtung zum Auslesen bestimmter biologischer Zellen von anderen Zellen und zum Beobachten zumindest einer bestimmten Eigenschaft der ausgelesenen Zellen, wobei die Vorrichtung umfaßt:

Einen im wesentlichen ebenen Träger (1) vorgegebener Dicke mit oberen und unteren Oberflächen und mit einer geordneten Anordnung von sich dadurch erstreckenden Öffnungen (2), wobei die Öffnungen eine vorbestimmte Konfiguration mit vorbestimmten Abmessungen an den oberen und unteren Oberflächen aufweisen, die als Ober- bzw. Bodenabmessungen bezeichnet werden, wodurch, wenn biologische Zellen auf die obere Oberfläche aufgebracht werden, nur ausgewählte Zellen vorgewählter Abmessungen in den Öffnungen im wesentlichen zurückgehalten werden

mit im wesentlichen einer Zelle pro Öffnung, dadurch gekennzeichnet, daß die Oberabmessung jeder Öffnung größer ist als ihre kleinste innere Querschnittsabmessung und die Dicke des Trägers und die Oberabmessungen jeder Öffnung von der Größenordnung des Druchmessers der gewählten zellen sind.

2. Vorrichtung nach Anspruch 1, dadurch gekennzeichnet, daß Ober- und Bodenabmessungen jeder Öffnung im Bereich von Mikrometern liegen.

3. Vorrichtung nach Anspruch 1, dadurch gekennzeichnet, daß die Öffnungen (2) so bemessen sind, daß sie Lymphozyten zurückhalten, eine pro Öffnung.

4. Vorrichtung nach einem oder mehreren der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß die genaue Lage jeder Öffnung bestimmbar ist durch ihre Position mit Bezug auf Koordinaten von X- und Y-Achsen des ebenen Trägers.

5. Vorrichtung nach einem oder mehreren der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß weiterhin erste Gerüstmittel (10, 40) vorgesehen sind, um den Träger an einer bestimmten Stelle darauf zu stützen, wodurch jede Öffnung des Trägers an einer bekannten Stelle angeordnet ist, die als eine Adresse mit Bezug auf die Gerüstmittel definiert werden kann, Instrumente (Fig. 4, 5, 6b) um zumindest eine Zelleigenschaft zu beobachten und/oder zu messen und Steuermittel (94), um die Relativbewegung und die Arbeit der Instrumente mit Bezug auf den Träger zu steuern und dadurch die Beobachtung und/oder Messung zumindest einer Zelleigenschaft einer Zelle in einer beliebigen der Öffnungen, ausgehend von ihrer Adresse, zu ermöglichen.

6. Vorrichtung nach Anspruch 5, dadurch gekennzeichnet, daß die Instrumente optische Untersuchungsmittel (86, 87) umfassen, um die optischen Eigenschaften einer beliebigen der Zellen zu bestimmen.

7. Vorrichtung nach Anspruch 5, dadurch gekennzeichnet, daß die ersten Gerüstmittel (10) Einrichtungen aufweisen, um eine Vielzahl von voneinander entfernt angeordneten Trägern zu stützen, wobei jeder Träger in seinen Öffnungen Zellen aufweist und zweite Gerüstmittel (11), die selektiv in Bezug auf die ersten Gerüstmittel anordbar sind, um die Zellen auf jedem der voneinander entfernt angeordneten Träger gleichzeitig durch eine vorgewählte Materie stimulieren zu können, wobei die Steuermittel (94) dazu angepaßt sind, eine Relativbewegung der Instrumente zu steuern mit Bezug auf einen beliebigen der Träger, und dadurch die Beobachtung und/oder Messung zumindest einer Zelleigenschaft einer Zelle in irgendeiner speziellen der Öffnungen zur ermöglichen, ausgehend von ihrer Adresse.

8. Vorrichtung nach Anspruch 7, dadurch gekennzeichnet, daß die ersten und zweiten Gerüstmittel mit Bezug aufeinander angeordnet werden können, wodurch die Zellen angeregt werden können, indem sie durch entweder das

Ober- oder das Unterende der Öffnungen befeuchtet werden.

9. Vorrichtung nach Anspruch 7, dadurch gekennzeichnet, daß die zweiten Gerüstmittel Einrichtungen aufweisen, um verschiedene Anregungsmittel darin aufzuweisen, wodurch Zellen in zumindest einigen der Öffnungen von im Abstand voneinander angeordneten Trägern gleichzeitig angeregt werden können durch die verschiedenen Anregungsmittel.

10. Vorrichtung nach einem oder mehreren der Ansprüche 1 bis 6, gekennzeichnet durch Einrichtungen, die eine Kraft aufbringen können, um beliebige der Zellen an die Öffnungen anzuziehen oder von diesen abzustoßen.

11. Vorrichtung nach Anspruch 10, dadurch gekennzeichnet, daß diese kraftaufbringenden Mittel Vorrichtungen umfassen, welche in einem vorgegebenen Muster, bezogen auf die Öffnungen, angeordnet sind, um eine oder mehrere Zellen von den Öffnungen abzustoßen oder zu diesen anzuziehen durch elektromagnetische Mittel (101).

12. Vorrichtung nach Anspruch 10, dadurch gekennzeichnet, daß diese Kraftmittel Vorrichtungen aufweisen (Fig. 11), um einen Druckunterschied aufzubauen über zumindest einem bestimmten Abschnitt des oder der Träger.

13. Vorrichtung nach Anspruch 10, dadurch gekennzeichnet, daß die Kraftmittel Vorrichtungen umfassen (115, 116, 117), um das Anhaften der Zellen in den Öffnungen zu verbessern.

14. Vorrichtung nach einem oder mehreren der Ansprüche 10 bis 13, dadurch gekennzeichnet, daß die ersten Gerüstmittel (10) Einrichtungen aufweisen, um eine Vielzahl von im Abstand zueinander angeordneten Trägern zu stützen, wobei jeder Träger Zellen in seinen Öffnungen aufweist, und zweite Gerüstmittel (11), die selektiv, bezogen auf die ersten Gerüstmittel, angeordnet, werden können, um eine gleichzeitige Anregung der Zellen auf jedem der im Abstand voneinander angeordneten Träger durch eine vorgewählte Materie zu ermöglichen, mit Steuermitteln (94), die angepaßt sind, um eine Relativbewegung der Instrumente mit Bezug auf einen beliebigen der Träger zu steuern und dadurch die Beobachtung und/oder Messung zumindest einer Zelleigenschaft einer Zelle in irgendeiner bestimmten der Öffnungen, aufgrund ihrer Adresse, zu ermöglichen.

15. Vorrichtung nach Anspruch 14, dadurch gekennzeichnet, daß die ersten und zweiten Gerüstmittel mit Bezug zueinander angeordnet werden können, wodurch die Zellen angeregt werden können, indem sie entweder durch das Bodenende oder das obere Ende der Öffnungen befeuchtet werden.

16. Vorrichtung nach Anspruch 14, dadurch gekennzeichnet, daß die zweiten Gerüstmittel Einrichtungen aufweisen, um darin verschiedene Anregungsmittel zu enthalten, wodurch Zellen in zumindest einigen der Öffnungen der im Abstand voneinander angeordneten Träger gleichzeitig durch die verschiedenen Anregungs-

mittel angeregt werden können.

17. Verfahren zur Auslese besonderer biologischer Zellen von anderen Zellen, um die Beobachtung von zumindest einer ausgewählten Eigenschaft der ausgewählten biologischen Zellen zu vereinfachen, wobei diese Methode umfaßt, im wesentlichen mit den Zellen, einschließlich der ausgewählten interessanten Zellen, die obere Oberfläche zu bedecken, eines im wesentlichen ebenen Trägers von einer vorgewählten Dicke, und der obere und untere Oberflächen aufweist und eine geordnete Anordnung von Öffnungen dadurch aufweist, wobei diese Öffnungen eine vorgewählte Konfiguration mit vorgewählten Abmessungen an den oberen und unteren Oberflächen aufweisen und wobei die Methode einen Schritt beinhaltet, die obere Oberfläche des Trägers zu waschen, um Zellen zu entfernen, die nicht von den Öffnungen gehalten werden, wodurch lediglich ausgewählte Zellen von vorgewählten Abmessungen im wesentlichen in den Öffnungen zurückgehalten werden mit im wesentlichen einer Zelle pro Öffnung, dadurch gekennzeichnet, daß die obere Abmessung jeder Öffnung größer als ihre kleinste innere Querschnittsabmessung und sowohl die Dicke des Trägers als auch die oberen Abmessungen jeder Öffnung in der Größenordnung der Durchmesser der ausgewählten Zellen geschaffen wird und daß der Schritt, eine beliebige Zelle zu der Öffnung anzuziehen, in welcher sie gehalten werden soll oder bei dem die Zelle von der Öffnung, in der sie gehalten wird, abgestoßen wird, eine Funktion einer bekannten Zelleigenschaft ist.

18. Verfahren nach Anspruch 17, dadurch gekennzeichnet, daß es weiterhin einen Schritt aufweist, die Zellen in den Trägeröffnungen nach einer vorherigen Anregung so zu behandeln, daß sie im wesentlichen in ihren ursprünglichen Zustand zurückversetzt werden.

19. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß es weiterhin die Schritte aufweist, die Zellen in den Trägeröffnungen zu neutralisieren nach einer vorhergehenden Anregung und sie dann mit einer anderen Materie wieder anzuregen.

20. Verfahren nach einem oder mehreren der Ansprüche 17 bis 19, gekennzeichnet durch einen Schritt, ausgewählte Zellen auf dem Träger zu vervielfältigen, um ihre Anzahl zu erhöhen.

21. Verfahren nach Anspruch 17, gekennzeichnet durch die Schritte, ausgewählte Zellen von den Trägeröffnungen abzustoßen und sie anschließend zu vervielfältigen, um ihre Anzahl zu erhöhen.

22. Verfahren nach Anspruch 17, gekennzeichnet, durch ferner den Schritt, zumindest eine Zelle zu töten, welche nicht unter die ausgewählten Zellen fällt, während die Zelle an Ort und Stelle befindlich ist, nämlich in einer Öffnung des Trägers.

23. Verfahren nach Anspruch 22, gekennzeichnet durch ferner einen Schritt, die lebenden Zel-

len an Ort und Stelle auf dem Träger zu vervielfältigen.

24. Verfahren nach Anspruch 23, dadurch gekennzeichnet, daß es ferner einen Schritt aufweist, den Vervielfältigungsprozeß von zumindest einer Zelle zu beobachten, welche sich an Ort und Stelle vervielfältigt.

25. Verfahren nach einem oder mehreren der Ansprüche 17 bis 24, dadurch gekennzeichnet, daß die genaue Anordnung jeder Öffnung identifizierbar ist durch ihre Lage in Bezug auf Koordinaten von X- und Y-Achsen des ebenen Trägers.

26. Verfahren nach einem oder mehreren der Ansprüche 17 bis 25, dadurch gekennzeichnet, daß der anziehende oder abstoßende Schritt durch elektromagnetische Mittel erreicht wird.

27. Verfahren nach einem oder mehreren der Ansprüche 17 bis 25, dadurch gekennzeichnet, daß der anziehende oder abstoßende Schritt durch Mittel bewirkt wird, welche eine Druckdifferenz über zumindest einen ausgewählten Abschnitt des Trägers erzeugen.

28. Verfahren nach einem oder mehreren der Ansprüche 17 bis 25, dadurch gekennzeichnet, daß die Haftung der Zellen in den Öffnungen verbessert wird durch zweckdienliche Mittel.

Revendications

1. Appareil pour sélectionner des cellules biologiques particulières parmi d'autres cellules et pour observer au moins une propriété choisie des cellules biologiques sélectionnées, ledit appareil comprenant un support (1), sensiblement plan, d'une épaisseur présélectionnée, et définissant des surfaces supérieure et inférieure, et comprenant un réseau ordonné d'ouvertures (2) qui le traversent, lesdites ouvertures présentant une configuration présélectionnée avec des dimensions présélectionnées au niveau des surfaces de sommet et de fond, pouvant être définies respectivement comme dimensions de sommet et de fond, d'où il résulte que, lorsque les cellules biologiques sont disposées sur ladite surface de sommet, seules des cellules sélectionnées de dimensions présélectionnées sont maintenues sensiblement dans lesdites ouvertures à raison de pratiquement une cellule par ouverture, caractérisé par le fait que la dimension de sommet de chaque ouverture est supérieure à sa plus petite dimension interne en coupe transversale, et à la fois l'épaisseur dudit support et les dimensions de sommet de chaque ouverture sont de l'ordre des diamètres des cellules sélectionnées.

2. Appareil selon la revendication 1, caractérisé en ce que les dimensions de sommet et de fond de chaque ouverture sont de l'ordre de micromètres.

3. Appareil selon la revendication 1, caractérisé en ce que les ouvertures sont dimensionnées pour maintenir des lymphocytes, à raison d'un par ouverture.

4. Appareil selon l'une des revendications 1 à 3, caractérisé en ce que l'emplacement précis de

chaque ouverture est identifiable par sa position par rapport aux coordonnées sur les axes des x et des y dudit support plan.

5. Appareil selon l'une des revendications précédentes, caractérisé en ce qu'il comporte en outre des premiers moyens porteurs (10; 40) pour supporter ledit support à un emplacement défini sur ceux-ci, d'où il résulte que chaque ouverture dudit support est située à un emplacement connu, pouvant être défini comme une adresse, par rapport auxdits moyens porteurs, des moyens d'instrumentation (figures 4, 5, 6B) pour observer et/ou mesurer au moins une propriété cellulaire, et des moyens de contrôle (94) pour contrôler le mouvement relatif et le fonctionnement desdits moyens d'instrumentation par rapport audit support, et permettant ainsi d'effectuer l'observation et/ou la mesure d'au moins une propriété cellulaire d'une cellule dans n'importe quelle ouverture particulière parmi lesdites ouvertures, sur la base de son adresse.

6. Appareil selon la revendication 5, caractérisé en ce que lesdits moyens d'instrumentation comprennent des moyens de balayage optique (86, 87) pour déterminer des propriétés optiques de n'importe laquelle parmi lesdites cellules.

7. Appareil selon la revendication 5, caractérisé en ce que lesdits premiers moyens porteurs (10) comprennent des moyens pour supporter une pluralité de supports espacés les uns des autres, chaque support présentant des cellules dans ses ouvertures, et des seconds moyens porteurs (11) que l'on peut positionner de façon sélective par rapport auxdits premiers moyens porteurs, pour permettre aux cellules présentes sur chacun desdits supports espacés les uns des autres d'être stimulées de façon simultanée par une matière présélectionnée, lesdits moyens de contrôle (94) étant capables de diriger le mouvement relatif desdits moyens d'instrumentation par rapport à l'un quelconque parmi lesdits supports, et permettant ainsi d'effectuer l'observation et/ou la mesure d'au moins une propriété cellulaire d'une cellule dans n'importe quelle ouverture particulière parmi lesdites ouvertures, sur la base de son adresse.

8. Appareil selon la revendication 7, caractérisé en ce que lesdits premiers et seconds moyens porteurs sont aptes à être positionnés les uns par rapport aux autres d'où il résulte que lesdites cellules sont stimulables en étant humectées par, ou bien l'extrémité de sommet, ou bien l'extrémité de fond, desdites ouvertures.

9. Appareil selon la revendication 7, caractérisé en ce que lesdits seconds moyens porteurs comprennent des moyens pour contenir différentes matières stimulantes à l'intérieur de ceux-ci, d'où il résulte que des cellules dans au moins quelques-unes des ouvertures desdites supports espacés les uns des autres peuvent être stimulées de façon simultanée par lesdites différentes matières stimulantes.

10. Appareil selon l'une des revendications 1 à 6, caractérisé en ce qu'il comprend également des moyens produisant une force pour attirer ou

expulser n'importe laquelle parmi lesdites cellules vers les ou à partir des ouvertures.

11. Appareil selon la revendication 10, caractérisé en ce que lesdits moyens produisant une force comprennent des moyens disposés selon un motif prédéterminé par rapport auxdites ouvertures pour attirer ou expulser une ou plusieurs desdites cellules vers les ou à partir des ouvertures par des moyens électromagnétiques (101).

12. Appareil selon la revendication 10, caractérisé en ce que lesdits moyens produisant une force comprennent des moyens (figure 11) pour produire une différence de pression à travers au moins une partie sélectionnée dudit (ou desdits) support(s).

13. Appareil selon la revendication 10, caractérisé en ce que lesdits moyens produisant une force comprennent des moyens (115, 116, 117) pour augmenter l'adhésion des cellules dans lesdites ouvertures.

14. Appareil selon l'une des revendications 10 à 13, caractérisé en ce que lesdits premiers moyens porteurs (10) comprennent des moyens pour supporter une pluralité de supports espacés les uns des autres, chaque support présentant des cellules dans ses ouvertures, et des seconds moyens porteurs (11) que l'on peut positionner de façon sélective par rapport auxdits premiers moyens porteurs, pour permettre aux cellules présentes sur chacun desdits supports espacés les uns des autres d'être stimulées de façon simultanée par une matière présélectionnée, lesdits moyens de contrôle (94) étant adaptés pour diriger le mouvement relatif desdits moyens d'instrumentation par rapport à n'importe lequel parmi lesdits supports, et permettant ainsi d'effectuer l'observation et/ou la mesure d'au moins une propriété cellulaire d'une cellule dans n'importe quelle ouverture particulière parmi lesdites ouvertures, sur la base son adresse.

15. Appareil selon la revendication 14, caractérisé en ce que lesdits premiers et seconds moyens porteurs sont aptes à être positionnés les uns par rapport aux autres, d'où il résulte que lesdites cellules sont stimulables en étant humectées par, ou bien l'extrémité de sommet ou bien l'extrémité de fond, desdites ouvertures.

16. Appareil selon la revendication 14, caractérisé en ce que lesdits seconds moyens porteurs comprennent des moyens pour contenir différentes matières stimulantes à l'intérieur de ceux-ci, d'où résulte que des cellules présentes dans au moins quelques-unes des ouvertures desdits supports espacés les uns de autres, peuvent être stimulées de façon simultanée par lesdites différentes matières stimulantes.

17. Procédé pour sélectionner des cellules biologiques particulières parmi d'autres cellules pour faciliter l'observation d'au moins une propriété choisie des cellules biologiques sélectionnées, ledit procédé consistant à couvrir pratiquement, avec les cellules, y compris les cellules intéressantes sélectionnées, la surface supérieure d'un support sensiblement plan, d'une

épaisseur présélectionnée, et définissant des surfaces supérieure et inférieure, et comprenant un réseau ordonné d'ouvertures qui le traversent, lesdites ouvertures présentant une configuration présélectionnée avec des dimensions présélectionnées au niveau des surfaces de sommet et de fond, et ledit procédé comprenant l'étape consistant à laver ladite surface supérieure dudit support pour retirer les cellules non supportées dans lesdites ouvertures, d'où il résulte que seules des cellules sélectionnées de dimensions présélectionnées sont maintenues sensiblement dans lesdites ouvertures à raison de pratiquement une cellule par ouverture, caractérisé par le fait que l'on prévoit la dimension de sommet de chaque ouverture supérieure à sa plus petite dimension interne en coupe transversale, et à la fois l'épaisseur dudit support et les dimensions de sommet de chaque ouverture, de l'ordre des diamètres des cellules sélectionnées, et l'étape consistant à attirer toute cellule vers l'ouverture dans laquelle elle doit être supportée, ou à expulser toute cellule de l'ouverture dans laquelle elle est supportée, en fonction d'une propriété cellulaire connue.

18. Procédé selon la revendication 17, caractérisé en ce qu'il comprend en outre l'étape consistant à traiter les cellules dans les ouvertures du support après une stimulation préalable pour les faire retourner sensiblement à leur état précédent.

19. Procédé selon la revendication 18, caractérisé en ce qu'il comprend en outre les étapes consistant à neutraliser les cellules dans les ouvertures du support après une stimulation préalable et, ensuite, à stimuler les cellules avec une matière différente.

20. Procédé selon l'une des revendications 17 à 19, caractérisé en ce qu'il comprend l'étape consistant à faire se multiplier des cellules sélectionnées sur le support de façon à augmenter leur nombre.

21. Procédé selon la revendication 17, caractérisé en ce qu'il comprend les étapes consistant à expulser les cellules sélectionnées des ouvertures du support et, ensuite, à les faire se multiplier de façon à augmenter leur nombre.

22. Procédé selon la revendication 17, caractérisé en ce qu'il comprend en outre l'étape consistant à tuer au moins une cellule qui n'est pas parmi les cellules sélectionnées alors que la cellule se trouve *in situ*, à savoir dans une ouverture de support.

23. Procédé selon la revendication 22, caractérisé en ce qu'il comprend en outre l'étape consistant à faire se multiplier les cellules vivantes sur le support *in situ*.

24. Procédé selon la revendication 23, caractérisé en ce qu'il comprend en outre l'étape consistant à observer le processus de multiplication d'au moins une cellule qui se multiplie *in situ*.

25. Procédé selon l'une des revendications 17 à 24, caractérisé en ce que l'emplacement précis de chaque ouverture est identifiable par sa posi-

tion par rapport aux coordonnées sur les axes des x et des y dudit support plan.

26. Procédé selon l'une des revendications 17 à 25, caractérisé en ce que ladite étape d'attraction et/ou d'expulsion est effectuée par des moyens électromagnétiques.

27. Procédé selon l'une des revendications 17 à 25, caractérisé en ce que ladite étape d'attraction

et/ou d'expulsion est effectuée par des moyens pour produire une différence de pression à travers au moins une partie choisie dudit support.

28. Procédé selon l'une des revendications 17 à 25, caractérisé en ce que l'adhésion des cellules dans lesdites ouvertures est augmentée par des moyens adaptés à ce but.

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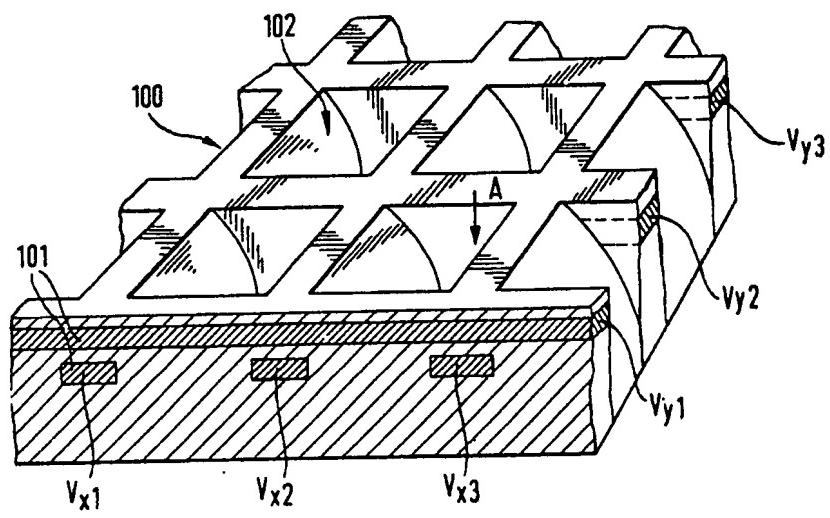
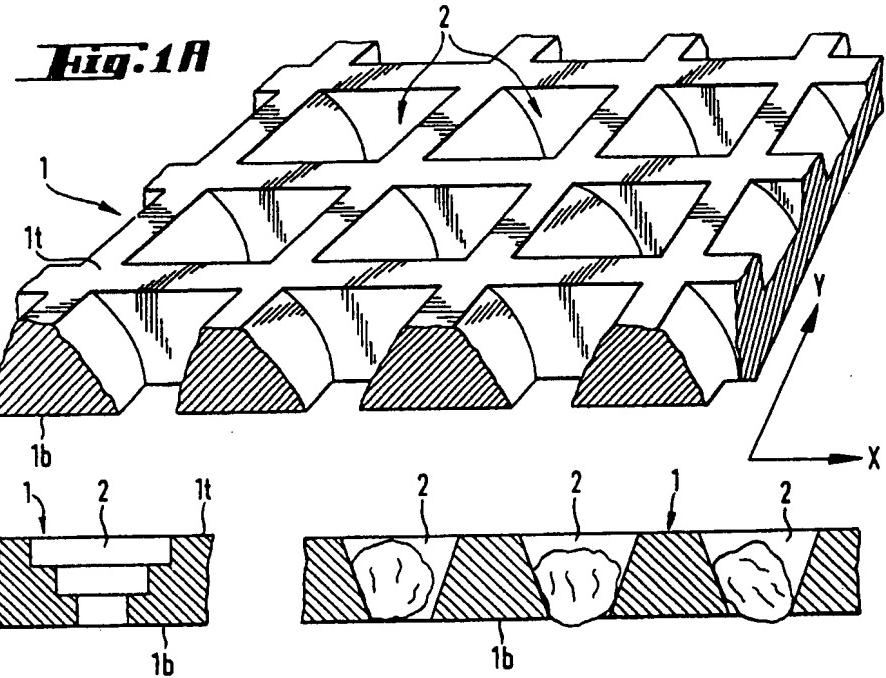


Fig. 17

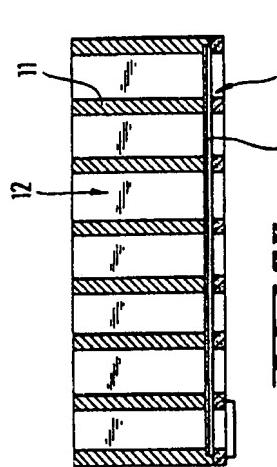


Fig. 2B

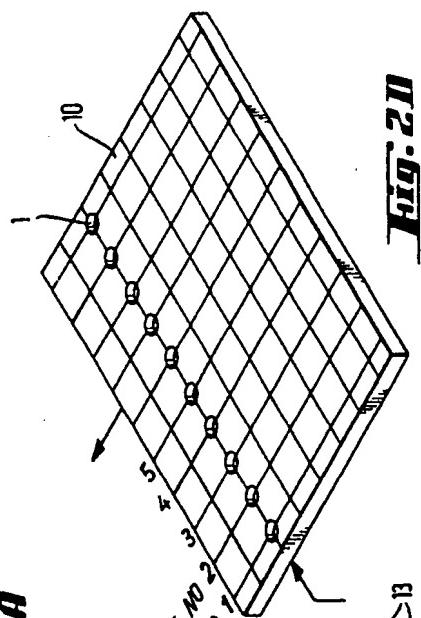


Fig. 2D

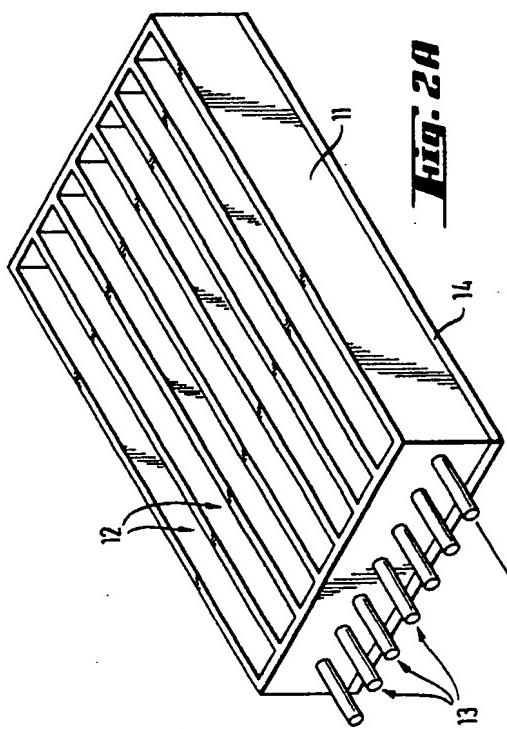


Fig. 2A

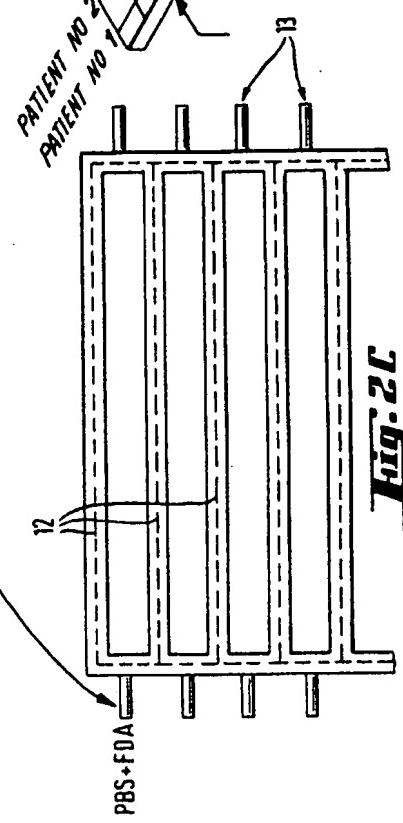


Fig. 2C

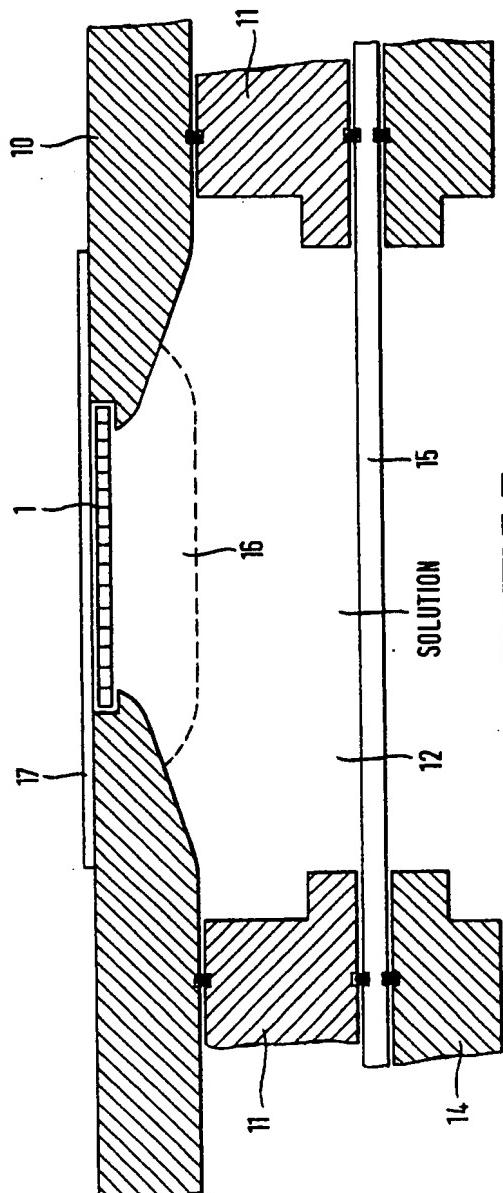


Fig. 3A

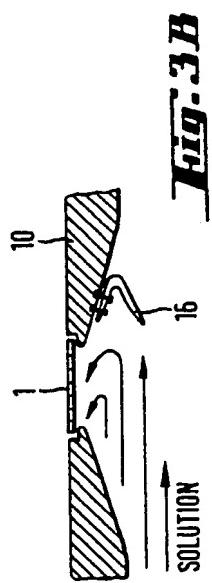
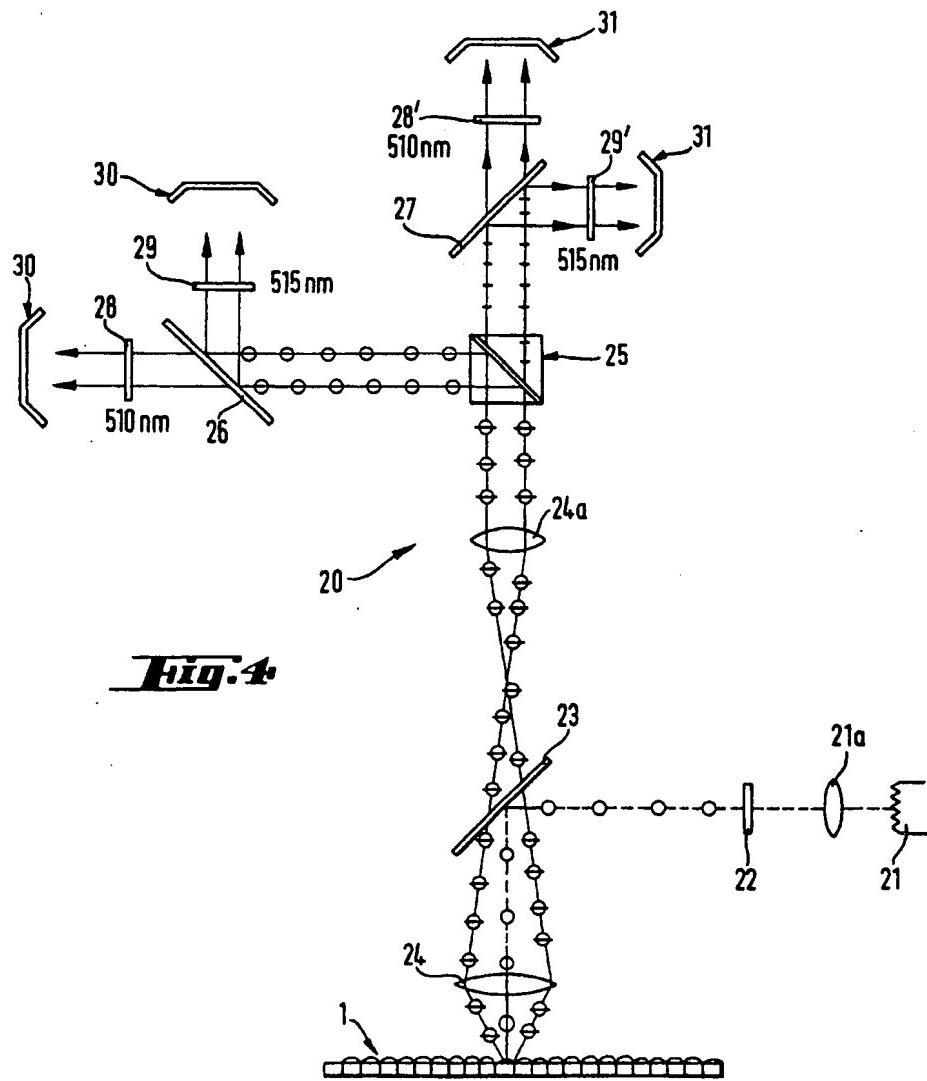
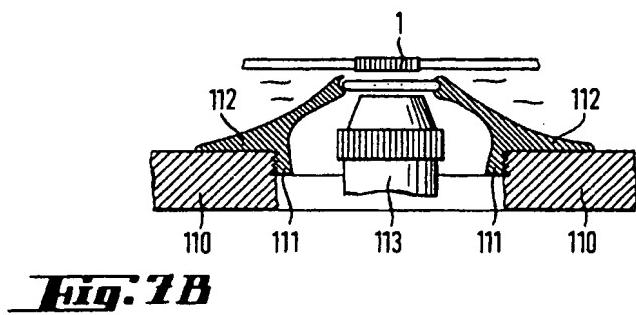
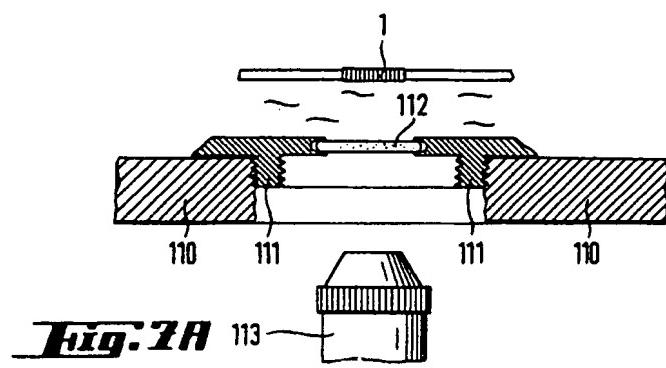
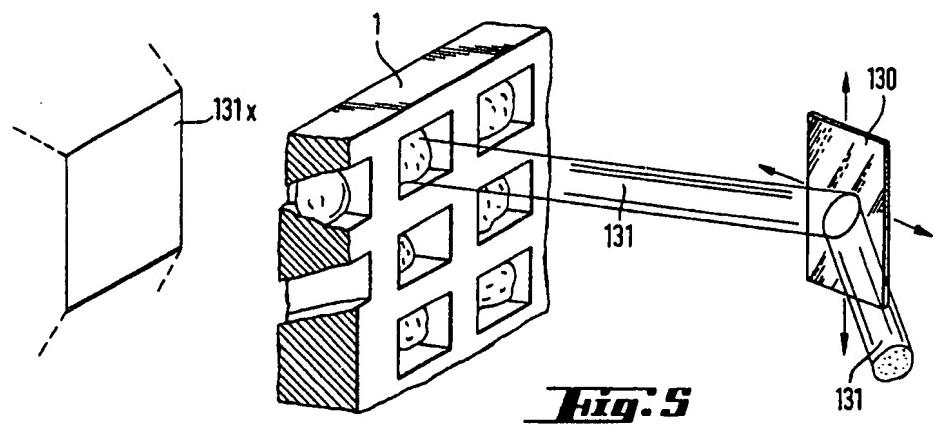


Fig. 3B



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Fig. 6B

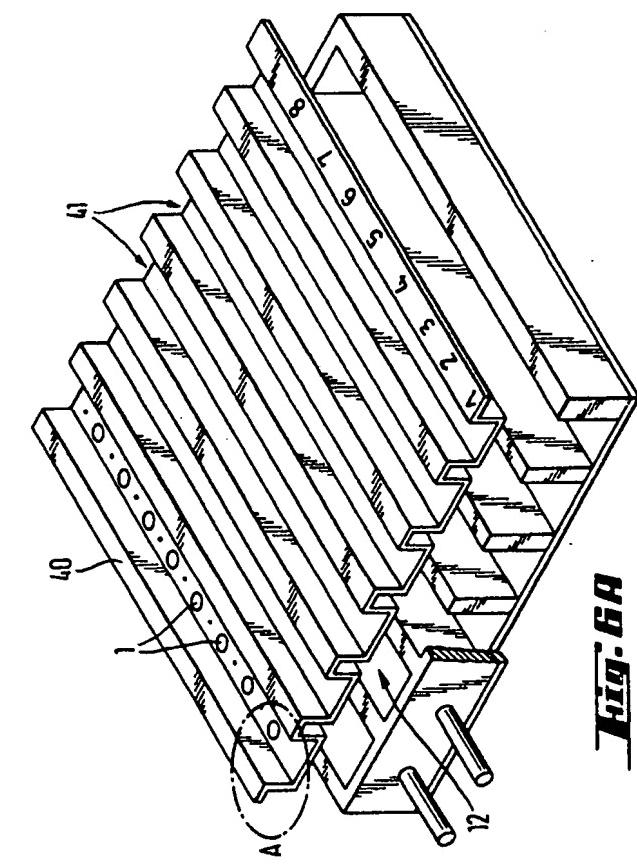
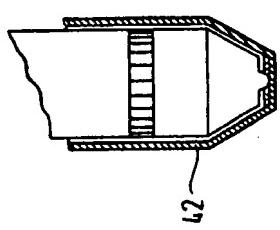


Fig. 6A

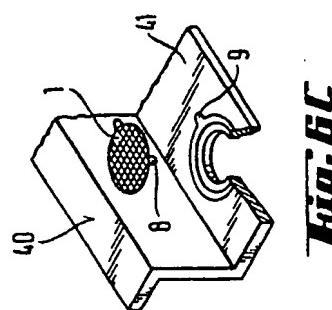


Fig. 6C

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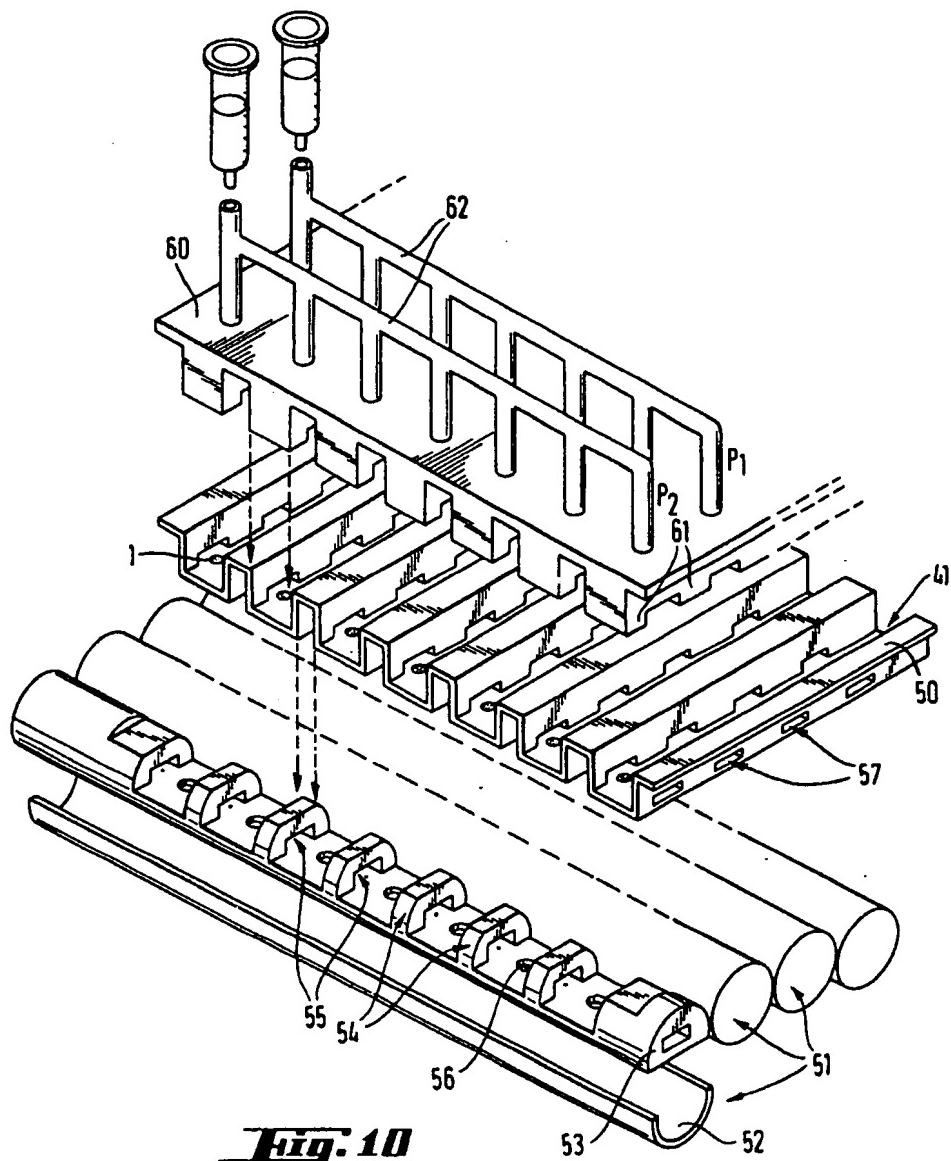


Fig. 10

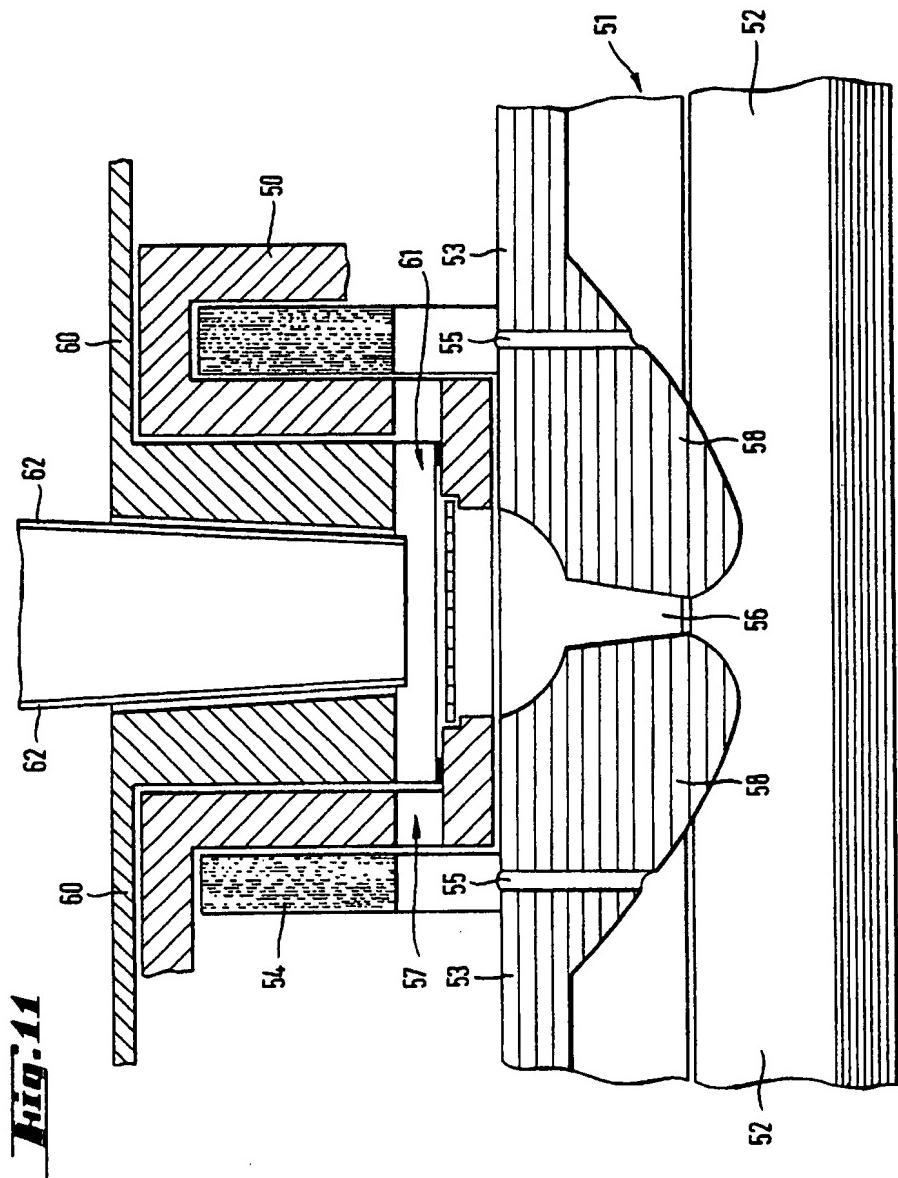


Fig. 11

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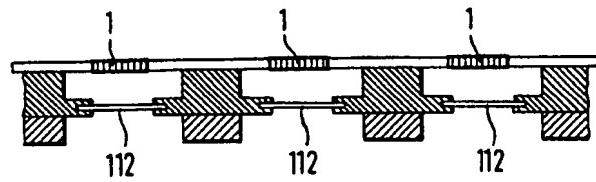


Fig. 8

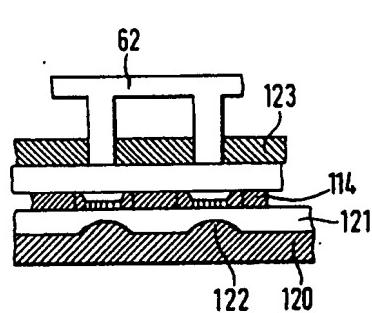
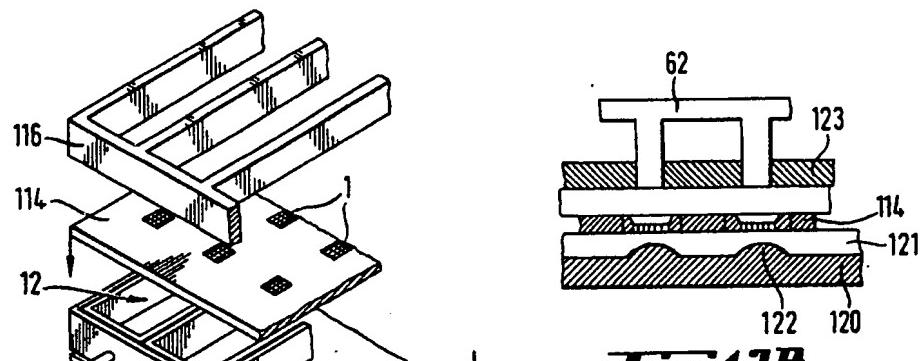
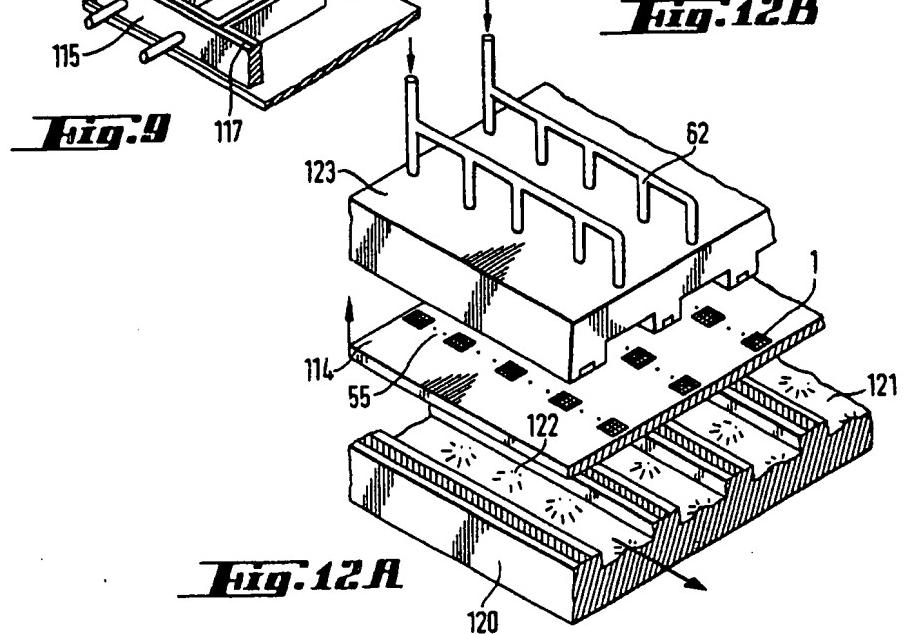


Fig. 12B



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Fig.13A

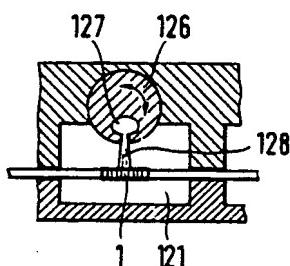
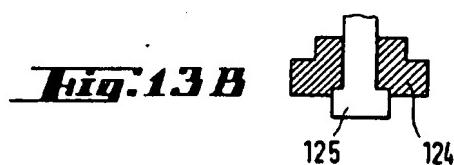
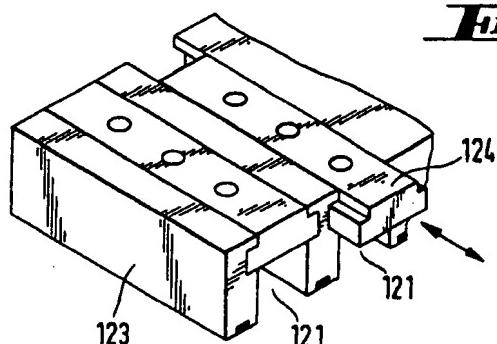
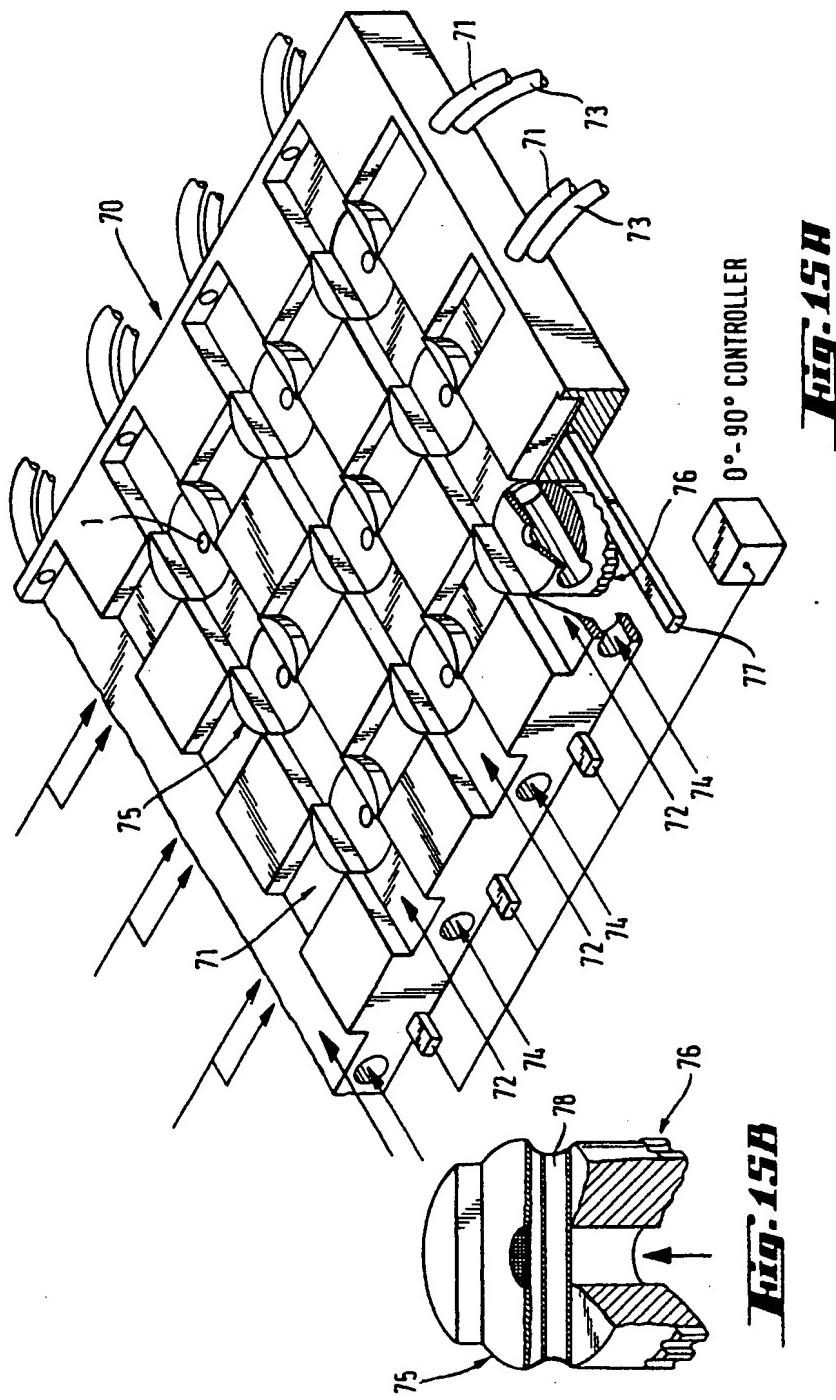
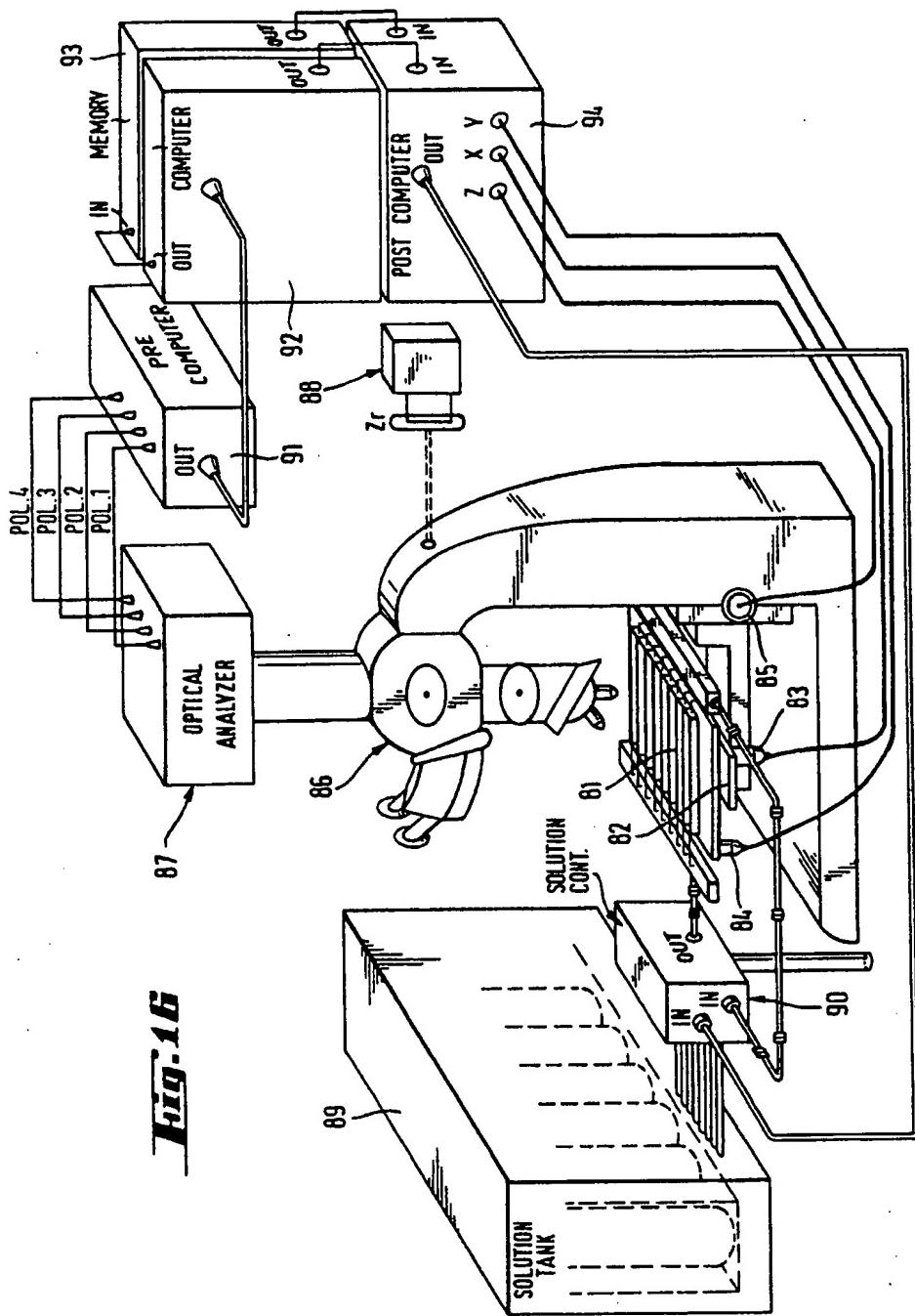
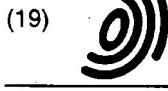


Fig.14

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(19)

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(54) Process for cell capture and recovery

Verfahren zur Fixierung und Rückgewinnung von Zellen

Procédé de saisie et de récupération de cellules

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Remarks:

Divisional application 95118035.5 filed on 16/11/95.

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Description

[0001] The subject field concerns cellular separations employing devices having specificity for cell surface proteins. Particularly, the cellular source will be blood, spinal fluid, bone marrow, tumor homogenates, lymphoid tissue and the like.

[0002] There are numerous situations where it is of interest to isolate a specific class of cells or to remove a particular set of cells from a mixture of cells. Techniques which have been employed include fluorescence cell sorting, magnetic immunobeads, complement-mediated lysis, affinity chromatography, centrifugal elutriation and polystyrene panning of cells. Cells having substantial density differences, such as that between platelets and red cells can be grossly fractionated by gradient centrifugation methodologies. However, mammalian cells with equivalent densities, such as tumor cells, lymphocyte subsets, granulocytes, or stem cells, require some form of separation using molecular recognition of surface markers which correlate with their phenotype. Similarly, such molecular mechanisms are required to separate viruses and bacteria from one another in complex mixtures. Each of the aforementioned methods have serious drawbacks for many applications, where there is interest in isolation or removal of particular subsets of cells.

[0003] Disadvantages of fluorescence activated cell sorting for recovery of viable sorted cells are the slowness of the procedure, the fact that the isolated cells are coated with antibody, and the limited amounts of cells which can be obtained by the procedure.

[0004] With immunobeads, it is difficult to recover the cells from the beads after separation; the cells are frequently coated with antibody and magnetic beads, and distinct separations are only difficultly achieved. Complement mediated lysis is problematic for two reasons: first, depletion of target cells is incomplete, and second, non-target cells can be adversely affected by exposure to complement and the toxic by-products of target cell lysis. Affinity chromatography of cells using, for example, Sephadex G-10 coupled to antibody, suffers from poor recovery and inefficient depletion of target cells. Centrifugal elutriation is not capable of separating different phenotypic subpopulations of cells of like size.

[0005] The last methodology, panning developed by Wysocki and Sato, PNAS, 75:2844, (1978), utilizing passively adsorbed antibody on polystyrene, is particularly inadequate. Only low recoveries can be achieved, and the process suffers from lack of specificity and contamination of the separated cells with antibody.

[0006] As the immune defense system becomes elucidated, it is increasingly evident that subsets of cells can have relatively narrow ranges of activities. Thus, subsets can be specialized for response to a particular disease, such as neoplasia, infection, viral or cellular, etc., response to transplants, and the like. It is therefore of great interest to be able to identify and purify these subsets of cells, not only to understand their action, but also to use these cells for prophylactic and therapeutic purposes. In order to achieve the desired results, it is necessary that substantially pure populations of the desired subset or subsets of cells can be obtained. Furthermore, the cells should be: (1) free of antibodies on their surface, (2) viable, (3) capable of fulfilling their normal function and (4) responsive to activation by biologicals in the same manner as normal cells in their normal environment.

[0007] According to the present invention, a method is provided for changing the cellular composition of a mixture of cells, the method comprising passing the fluid through a cell capture device so that the cells contact at least one internal flat polystyrene surface of the device which is a sheet, fibre, container wall or partition the surface having covalently bound at least one receptor specific for at least one ligand present on at least some of the cells in the fluid for sufficient time for the cells containing said ligand to specifically bind to the surface; and

removing cells which are not specifically bound without significantly disturbing specifically bound cells. The cells may be released from the receptors by either biological activation resulting in ligand shedding and release or physical means such as pipetting, mechanical vibration or ultrasonic sound, as appropriate. The cells can be numerically expanded, subjected to biologicals or other factors for differentiation and/or activation, or the like, and may be used for research, diagnostic, prophylactic and/or therapeutic purposes.

[0008] In the accompanying drawings:

Fig. 1 is a schematic drawing of a process to prepare cells from peripheral blood or bone marrow for the captured device according to this invention; and

Fig. 2 is a diagrammatic view of the inside of a cell capture device.

[0009] Methods are provided for isolating a particular population of biologically replicatable particles, particularly cells, from a mixture of particles by binding the population to a solid substrate through the intermediacy of one or more specific receptors. Optionally, the particles may then be treated in a variety of ways to affect the population size and/or characteristics of the captured particles. The cells may then be released from the support substantially free of receptor.

[0010] The method will involve contacting a source of particles with receptor bound to a support in a collection device, where the population of the receptor on the surface provides for a high binding density for the ligand(s) of interest. The conditions for the contact are such as to allow for sufficient time and a low degree of turbulence to permit the particles

to specifically bind to the receptor. After sufficient time for binding to occur, the medium may be removed and the surface washed to remove particles which are not specifically bound particles. Since the particles will normally be bound to the surface at a plurality of sites, so as to have a high binding avidity, the washing may be fairly vigorous to insure the substantially complete removal of all particles which are not specifically bound. The particles may then be subjected to a wide variety of conditions or treatments, usually involving contact with one or more reagents. Optionally, the medium containing the reagents may then be removed and the particles released from the receptors. Release may be achieved either by treatment with a combination of a mitogenic agent and a lymphokine or physical means such as pipetting, vibration or sonication. The particles may then be isolated and used for their intended purpose.

[0011] The device finds application in a number of different situations. The first is a situation in which one wishes to capture and remove undesirable cells from physiological fluids, thereby depleting the fluid of the undesirable cells for therapeutic benefit, or diagnostic or research applications. This may be illustrated by depleting bone marrow of certain T-lymphocytes to diminish graft-versus-host disease. The bone marrow, depleted of the unwanted cells, is immediately prepared for transplantation into the marrow recipient.

[0012] A second situation is to capture and recover certain cells from physiological fluids for research and diagnosis. Diagnostic applications may include capture and subsequent enumeration and description of captured (1) malignant cells from blood or other tissues, (2) viral or bacterially infected mammalian cells, (3) viruses or bacteria or parasites themselves from physiological fluids, (4) human fetal cells for karyotypic analysis from blood, (5) transplanted cells from blood as an index of recovery from bone marrow transplantation, and (6) immune competent cells with a particular surface marker, such as the presence of the IL-2 receptor, indicating a state of activation. For research, one may be interested in (1) the genetic analysis or modification of captured cells, (2) analysis and modification of the physiology of certain classes of cells that may be activated or suppressed by a particular disease process and (3) at the molecular level, the surface membrane compositional analysis and modification of cells involved in the pathogenesis of a particular disease.

[0013] The third situation lies in the capture and recovery of cells from physiological fluids for modification (activation) and return to the patient of origin for a desired therapeutic effect. The process involves cell capture and recovery, processing of the captured cells or depleted cell population which may result in numerical expansion and/or biological activation, and the subsequent recovery and use of these cells.

[0014] The third situation may be further divided into three levels of application. The first level is biological activation of the captured/recovered cells themselves. Activation is performed without further fractionation of the cells. For example, in the case of an AIDS patient, CD8 positive cells captured from peripheral blood can be expanded and activated for subsequent return to the patient of origin. The second level is selective activation. Captured and recovered cells are further processed or fractionated to provide a subset of captured cells, identified for example by antigen specificity, which are then activated and/or expanded. For example, certain antigen restricted subsets of the captured CD8 positive cells can be selected by certain co-culture conditions and concentrations of lymphokines, which allow only the desired subset to be expanded and activated. A third level is *in vitro* generation of antigen specific patient-unique cells for activation or suppression of the immune function. Exemplary of this situation is monocyte or B-cell capture and exposing the captured monocytes or B-cells to a patient-specific immune complex or other antigens under conditions which augment monocyte or B-cell antigen uptake, processing, and presentation along with increased surface MHC expression. One would then add a subset of effector or regulatory cells captured from the same patient to interact with the antigen-primed monocyte or B-cell. The process of antigen specific T-cell activation would occur, much in the manner that occurs in the lymph node of an intact animal or human. An additional example of cell modification made possible by the subject method is the introduction of exogenous genes via viral or other vectors or other means into the captured cells and the subsequent capture in a second device of the subpopulation of cells which express the exogenous gene.

[0015] The cellular source may be any mixture of cells. However, it is desired to have a predetermined population which may be defined by single or multiple markers or plurality of markers or ligands. Cellular sources of interest from animal hosts may include organs, such as blood, brain, kidney, spleen, heart, intestine, bone marrow, cerebral spinal fluid, lymphoid tissue, or the like, or neoplastic cells from any of the above organs. Other sources may be parasites, viruses or bacteria mixed with animal cells. The cells are employed in a flowable form, conveniently as a dispersion. Where the cells are not held together, as in blood, the blood will usually have red cells, platelets and plasma removed to provide for a mixture of white cells. Where the cells are held together by a membranous or other connecting material, the cells may be dispersed either mechanically or enzymatically in accordance with conventional techniques. The individual cells may then be dispersed in their appropriate nutrient medium for separation by the subject method.

[0016] With blood, red blood cells may be removed by agglutination, lysed with ammonium chloride, removed with lectins or by centrifugation in accordance with known ways. Platelets and red blood cells may also be removed by gradient density centrifugation, employing Ficoll or Leukoprep and isolating the buffy coat, by centrifugation or the like.

[0017] The various cell sources may be subjected to a variety of treatments in addition to those described above. In some situations, it may be desirable to concentrate the cells by any convenient means, followed by dispersion in an appropriate nutrient medium. In some situations it may be desirable to expand a particular population, where one can

selectively expand one group of cells as against another group of cells. For expansion, various mitogenic agents may be employed or interleukins, growth factors, or the like. These cells will then usually be concentrated by any convenient means to substantially remove the medium in which they have been isolated or maintained. Usually, these cells will comprise at least about 10 vol % of the dispersion to be used and not more than about 90 vol %, so as to provide a flowable dispersion. The concentration of cells introduced into the device is conveniently based upon the surface area of the derivatized polystyrene surface and will vary widely, depending upon the frequency of the target cell in the input cell suspension. Usually, the concentration will be at least 1×10^3 cells/cm² and not more than 1×10^{10} cells/cm², usually from about 1×10^5 cells/cm² to 1×10^7 /cm².

[0018] The separation device may take a wide variety of forms. For the most part, the device will be comprised of polystyrene surfaces, where the polystyrene is normally substantially free of cross-linking, less than about 0.5%, usually less than about 0.1%, preferably molded or extruded, so as to have a very smooth surface. Polystyrene surfaces of this nature allow for substantial uniformity of derivatization, where the orientation of the receptor provides for a high level of accessibility of binding sites. (It should be understood in referring to receptor, the term is entirely arbitrary. By receptor is intended a molecule which is able to specifically bind to a complementary molecule. Thus, for the purposes of this invention, the receptor may be a ligand, which includes both haptens and antigens, or a surface membrane protein which specifically binds to another molecule, such as an immunoglobulin, T-cell receptor, insulin receptor, etc., or a molecule which is found intracellularly, such as a steroid binding protein, or molecules which are found in body fluids, such as thyroxine binding globulin, lipoproteins, etc. Therefore, the membrane protein which binds specifically to the surface bound "receptor" is referred to arbitrarily as the "ligand." For convenience, they will be referred to jointly as complementary members of a specific binding pair.)

[0019] The functionalized polystyrene surface may be the surface of a wall, partition, sheet, hollow fiber, or the like. At least one surface is a flat surface. The device may take the form of a bottle, standard T flask, sheets, e.g., a bag or box with multiple separated sheets, cylindrical or serpentine sheets in a container, rectangular box, or the like. The choice of the device will depend upon convenience, the purpose of the separation, the interaction with other devices, the cell population of interest, the intended treatment, whether the population of interest is as a result of positive or negative selection, or the like.

[0020] The surface will be derivatized by substitution of the benzene ring of the polystyrene with an electrophilic reagent, particularly by a Friedel-Crafts reaction in a solvent which does not soften or dissolve the polystyrene. For this purpose, sulfolane finds particular application. Relatively mild conditions may be employed and the benzene may be derivatized with a variety of agents, such as nitro, which may be reduced to amino, halomethyl, which may be used to form an amino, hydroxy, or thiol group, or a substituted N-hydroxymethyl acetamide where the substituent is an active halogen or pseudohalogen. A description of the reaction may be found in EPA 88-304516.3.

[0021] The derivatized polystyrene surface may then be reacted with the receptor. Under the conditions of derivatization, it is found that a high percentage of the benzenes at the surface are derivatized, so that one may obtain a high density of receptor at the surface.

[0022] Depending upon the nature of the receptor, various reactions may be performed for bonding the receptor to the surface. Of particular interest is the bonding of proteins to the surface. Proteins can be bonded by contacting the proteins in an aqueous medium with the functionalized surface, having active halogen, activated carboxy groups, e.g., esters, or the like, under mild conditions for sufficient time for complete reaction. Any remaining unreacted functional groups may be blocked by using an appropriate small molecule blocking agent. For example, active halogen may be blocked with aliphatic amines, thiols with maleimide, or the like. In some situations, there may be no need to block excess reactive groups, since they will not interfere with the subsequent steps in the process. The surface may then be washed to remove the non-specifically bound receptor and evaluated to insure that appropriate receptor binding has occurred.

[0023] Depending upon the nature of the collection device, the contact with the cell containing medium will be varied. For example, with a roller bottle, one may introduce the medium into the roller bottle and then allow for slow revolution of the bottle over sufficient time for the cells to become bound. With a T-flask, or plates-in-a-bag/box configuration, one may allow the device to stand on a level surface or be slightly agitated on a shaking platform, followed by turning the device over and repeating the process on the other side. Similar techniques may be employed with other types of containers. Additionally, the device may be centrifuged to press the target cells to the contact surface.

[0024] Of particular interest, is a device which will be referred to as a collection bag/box. The bag/box will be a container of rigid or flexible walls containing polystyrene sheets superimposed or stacked one upon the other and separated from each other to allow for flow between the sheets. Packed cells as a result of concentration, e.g., gradient density centrifugation or centrifugation, would be allowed to flow into the bag/box which would be maintained in a horizontal position. The cellular dispersion would spread through the bag/box, so as to be in contact with substantially all of the receptor-coated polystyrene surface in the bag/box. After sufficient time for the cells to bind, the bag/box may be turned over so as to allow cells which are still dispersed or unbound to settle on the film surfaces which are now below them, so as to provide for efficient utilization of the surface. Alternatively, the bag/box may be centrifuged, once

on each side, to press the cells to the contact surfaces.

[0025] The contact time will vary widely, depending upon the concentration of the ligand on the cell surface, the binding affinity of the receptor, the concentration of cells in the medium, the nature of the collection device, and the like. Usually contact times will be at least about 5 min and not more than about 120 min, usually from about 15 to 60 min.

[0026] The cellular dispersion may be moved through the collection device by any convenient means. A pressure differential may be achieved through the collection device by means of pumping. Alternatively, gravity flow may provide for an appropriate flow rate. Any convenient technique which allows for a rate of flow of the cells permitting binding to the surface without significantly affecting their viability may be employed.

[0027] The subject devices can be sterilized using gamma or electron beam radiation, without adversely affecting the properties of the collection device. That is, the activity of the receptor is retained, while at the same time retaining the covalent nature of its bonding to the surface. Thus, when the collection device is in use, substantially none of the receptor bound to the surface is lost.

[0028] Once the cells have become bound to the surface, the collection device may be subject to a wide variety of treatments. Vigorous washing may be employed to remove non-adherent cells, since the adherent cells are bound firmly to the surface at a plurality of contacts. The wash medium may be pumped in and out, ligands flowed through the device, or other means of mild but relatively vigorous agitation. The wash solution may be deionized water, saline, phosphate buffered saline, nutrient medium, or the like. The particular wash solution which is employed will usually depend upon how the cells are to be used.

[0029] Where the cell isolation is concerned with removal of cells from the cell population; (cell depletion), the captured cells may be discarded and the depleted cell population harvested, subjected to any additional treatments, and then used for its intended purpose.

[0030] For the most part, the subject invention finds particular application for cells which have been isolated for subsequent use. Depending upon the intended use, as well as the nature of the cells, the cells may be subjected to a wide variety of treatments. Particularly, where one is concerned with the lymphoid or myeloid lineages, these cells may be treated to expand or modify the activity of a particular set or subset of cells. Thus, various factors may be added which result in the proliferation of the cells, activation of the cells, enhancement or reduction of one or more surface membrane proteins, and the like. Depending upon whether one wishes to have all cells bound during the treatment or allow for the formation of free cells, one can provide for an appropriate ratio of receptor to bound cells in the container. By having a large number of receptors compared to the initially bound cells, any progeny will also become bound and retained on the surface. This may serve as an additional resolution, since other cells which may have been present and expanded will not become bound and may be removed from the collection vessel.

[0031] For the most part, the cells of interest will be obtained from blood, bone marrow, solid tumors and lymphoid tissue. These cells may be divided into the lymphoid and myeloid lineages. The first lineage to be considered will be the lymphoid lineage. This lineage may be further broken down into categories of B-cells and T-cells. B-cells are identified by having sIg as a surface marker and rearranged germline DNA at the immunoglobulin locus. T-cells, for the most part, have CD2 and/or CD5 as surface markers and rearranged germline DNA at the T-cell receptor locus. The B- and T-cells will also include specific progenitor cells, although pluripotent stem cells will be discussed separately, and in the case of B-cells, plasma cells are also included.

[0032] Other specialized lymphoid cells which may be isolated by markers include: lymphokine activated killer (LAK) cells, natural killer (NK) cells, tumor infiltrating lymphocytes (TIL), antibody dependent cytotoxic cells (ADCC), cytotoxic T lymphocytes (CTL), etc.

[0033] In the myeloid lineage, one may be interested in isolating monocytes, macrophages, eosinophiles, basophils, polymorphonuclear leukocytes, dendritic cells, etc.

[0034] The B-cells may be expanded by treatment with various of the interleukins, 1-7 or others, when discovered, particularly IL-1, -2, -3, or the like. The B-cells may be selected by surface bound antigen, surface markers (e.g., CD20) or by specific binding to a soluble antigen, where such antigen may be added to the cells, so that those cells having a surface immunoglobulin which recognizes the antigen will bind the antigen to form a complex which is endocytosed and processed. A fragment of the antigen with the cell's MHC antigen will then be presented. By adding T-cells to the medium which are restricted by the B-cells, T-cells which recognize the antigen fragment will secrete lymphokines, resulting in proliferation of the B-cells. By providing for an excess of receptor on the solid surface or after release of the B-cells separating the cell population in a second collection device, one can substantially augment the number of B-cells and plasma cells which recognize the antigen of interest.

[0035] Alternatively, B-cell fusion partners (hybridoma cells) or other B-cells from any source can be selected by binding to a polystyrene surface which bears covalently bound antigen. Desired hybridoma or other B-cells bearing sIg reactive with polystyrene bound antigen will be captured on the polystyrene surface, allowing for antigen-specific selection of specific hybridoma or other B-cells. Captured cells can then be recovered and expanded according to the procedures described in the subject method. Alternatively T-cells or any cell containing a specific surface receptor can be captured by the polystyrene surface when said polystyrene surface contains said antigen covalently bound.

- [0036] Where one wishes to deplete a specific subset of B-cells, one may add the antigen conjugated to a toxin, employ antibodies specific for the surface immunoglobulin and complement or other selective cyto-toxic capability. In this way, one may selectively diminish the cells responsive to a particular antigen. Alternatively, antigen or a B-cell marker (e.g., CD-20) can be immobilized on the polystyrene and the targeted B-cell population captured on the surface. Particularly, where memory cells exist, one can reduce the humoral response by substantially depleting the memory cell population to a particular antigen.
- [0037] The T-cell population is more varied than the B-cell population as to function. One may divide the mature T-cell population into CD4 MHC Class II restricted cytotoxic, helper or suppressor cells and CD8 MHC Class I restricted cytotoxic and suppressor cells, where the cells have different functions and their expansion and depletion may be of interest.
- [0038] For either T-cell population (CD4 or CD8), it may be desirable to activate the T-cells which recognize a specific antigen. Many strategies can be used for this purpose. B-cells specific for a particular antigen may be exposed to that antigen and then used as antigen presenting cells to activate the particular antigen restricted T-cell subset. Alternatively, monocytes or macrophages may be employed as the antigen presenting cells. Macrophages may be preferred since they do not have the specificity of the B-cells for a particular antigen. Therefore, one would introduce monocytes and/or macrophages, which have been pre-treated or treated concomitantly with the antigen, to the bound T-cells to provide for expansion of those T-cells which recognize the antigen fragment when presented by the monocyte/macrophage in the context of the MHC.
- [0039] Biological activation of cells may be achieved as a result of a particular soluble or immobilized lymphokine, e.g., IL-2, or by use of a specific binding compound, such as an antibody. For example, T-cells may be selected using an anti-T-cell (e.g., CD-5) surface. The CD-5⁺ captured cells may then be released and introduced onto an activating anti-CD3 surface, or to a surface to which a lymphokine has been covalently bound. The cells will bind and become activated. After activation, the cells may be released by sonication, mechanical agitation or other convenient means and harvested.
- [0040] Of particular interest are stem cells, which may be obtained from bone marrow or peripheral blood. These stem cells may serve as the progenitors of one or more of the blood cell lineages. Isolation of the stem cells may be as a result of both depletion (negative selection) and/or positive selection. Thus, one may provide for a series of devices or device subsections where initially the receptors will bind to undesired cells for their removal of cells (negative selection) from the medium. The unbound cells may then be isolated, freed of the captured cells and further selected (positive selection) for cells with different markers associated with stem cells, leaving a bound population of cells, which may then be freed followed by further positive selection for a marker specific for a population which includes the stem cells. In this way other cells having the analogous final marker may be removed by the previous process step.
- [0041] Where cells other than blood cells are involved, cells of interest for isolation may include islets of Langerhans, glial cells, astrocytes, neurons, endothelial cells, epithelioid cells, stromal cells, stem cells, squamous cells, or the like.
- [0042] Substantially homogeneous populations, greater than about 95%, usually 98%, of cells may be achieved, where the cells may be in a quiescent or activated state. Cellular compositions may include any cellular population expressing a surface marker (ligand) recognized by the immobilized receptor. Such compositions include cells bearing any of the recognized leukocyte antigens of the CD (cluster designation series) or others recognized by monoclonal antibodies to specific cell surface ligands. Such compositions may include other blood cells, tumor cells, bacteria, viruses, or parasites similarly sharing a common surface marker. Virtually any cell population whose members share a surface ligand recognized by the immobilized receptor can constitute such a cellular composition.
- [0043] A great variety of autoimmune, neoplastic, infectious, metabolic, hematologic and immunologic diseases and conditions (the disease field) may be treated in accordance with this invention. Among autoimmune diseases are diabetes, lupus erythematosus, and rheumatoid arthritis. Among infectious diseases are localized and systemic infections due to gram positive cocci, gram negative cocci, gram negative bacilli, anaerobic bacteria, mycobacteria, fungi, viruses, protozoa, etc. Among neoplastic diseases are all solid and hematologic malignancies. Among metabolic diseases are atherosclerosis and septic shock. Among hematologic diseases are sickle-cell anemia and familial hypercholesterol anemia. Among immunologic diseases and conditions are organ transplantation and immunodeficiency conditions.
- [0044] These and other diseases or conditions may be addressed by the subject process as follows. By an alternative process, one may isolate immune complexes associated with the autoimmune infectious or neoplastic disease (see co-pending Application Serial No. 243,786, filed September 13, 1988). One can use the antigen obtained from the complexes to select for both B- and T-cells as described above which are activated by the particular antigen. Thus, one can remove blood from the host suffering from the autoimmune or neoplastic disease and either selectively deplete B- and/or T-cells associated with the disease or activate T- or B-cells to suppress the autoimmune disease or to detect and eliminate the neoplastic cells. In this way, one may provide for a remission, halt the progress of the disease, or the like.
- [0045] Alternatively, in cases of infection, autoimmune or neoplastic disease, one may provide for selection of B-

and T-cells reactive with the particular pathogen or disease antigen. In this case, one would wish to enhance the concentration of the B- and T-cells associated with the immune defense. Thus, complexes or antigens associated with the pathogen, autoimmune or neoplastic disease or the pathogen, autoreactive or neoplastic cell itself may be used to enhance the lymphoid cellular population associated with the defense against the disease. One may isolate the pathogen, autoreactive or neoplastic cell using the subject device, and use the isolated pathogen or cell as the immunogen or receptor, as defined above to capture the appropriate T- or B-cells active in the defense against the disease. Thus selected, these cells could be recovered, expanded, activated as described above for a subsequent return to the patient of origin. This technique may be used with a wide variety of diseases associated with viruses, e.g., AIDS, HTLV-I, or II, bacteria, protozoa, fungi, helminths, and the like.

[0046] In addition, the subject method may be used for prophylactic and diagnostic purposes in the disease field. The subject method will also find use in research for detecting B- and T-cell responses, investigating immune responses, identifying epitopes associated with autoimmune diseases, and ultimately used for gene therapy.

[0047] One may also use the device for producing monoclonal antibodies by activating B-cells, followed by immortalization of the B-cells usually by fusion with an appropriate fusion partner. In this way, one can immunize human lymphocytes against antigens one could not normally administer to a human host and provide for double selection, initially for B-cells generally, followed by selection for those specific B-cells which are capable of binding to the antigen. Thus, one can greatly concentrate B-cells specific for the antigen to greatly enhance the probability of obtaining monoclonal antibodies specific for the antigen.

[0048] The cells may be isolated from the collection device by different ways. Of particular interest is the use of a mitogenic agent, such as phytohemagglutinin (PHA), in conjunction with a compound having growth factor-like activity such as an interleukin or growth factor, e.g., interleukin-2 (IL-2), GM-CSF, etc. which results in release of the cells by shedding of the ligands on the cell surface bound to the receptor. The medium may be a standard tissue culture medium containing about 20 to 1000 units/ml IL-2 and about 0.1 to 5.0 µg/ml of phytohemagglutinin. Alternatively, the cells may be released by physical methods such as mechanical disruption, particularly shearing, such as by vibration, vigorous pipetting or by sonication using an ultrasonicator and placing the collection device in a water bath. Conveniently, a Crest ultrasonics model may be employed. See Menssen, et al., *J. Immunol. Methods* (1987) 104:1-6.

[0049] In order to further understand the invention the figures will now be considered. Fig. 1 is a diagrammatic flowchart of a process according to the subject invention using blood as the source of target cells. The drawing involves a first stage involving the separation vessel 10, where red blood cells and platelets are removed to provide a supernatant. The supernatant 12 is then transferred to a centrifuge 14 having tubes 16, where the supernatant 12 is centrifuged to concentrate target cells 20 in the tubes 16. The target cells 20 are then transferred to a feeding vessel 22, which feeds the target cells through valve 24 into cell capture device 26, also depicted in Fig. 2. This process is not limited by the example cited. Any commonly used method to remove red blood cells, platelets and plasma can be used to achieve target cell population 20 from peripheral blood or bone marrow. Alternatively, solid tissue may be disaggregated by enzymatic or physical means to achieve target cell population 20.

[0050] Cell capture device 26 has a plurality of polystyrene films or sheets 30 separated by supports 32. On the upper films or sheets 30 are indicated the presence of receptors 34 designated as R. The receptors 34 are only indicated on a few of the films or sheets, indicating that the receptors are on both sides of the film or sheet, although it should be understood that all of the films or sheets are coated on both sides with receptors. Alternatively, the cell capture device can be a T-flask, microtiter plate, multiwell plate, roller bottle, cell farm or any other polystyrene vessel all or part of whose internal polystyrene surface has receptor immobilized to it.

[0051] The cells enter the cell capture device 26 through conduit 36. When cell capture device 26 is substantially full, it is allowed to stand for sufficient time for the cells to settle and contact the receptors on the film or sheet below the liquid layer. After sufficient time for the cells to have settled and become attached, the cell capture device 26 may then be turned over so that cells which have not become specifically bound may settle on the reverse side of the films or sheets 30 and become bound to the receptors on that side. The cell capture device may then be washed by introducing a wash solution through conduit 36 and allowing it to exit through conduit 40, so as to remove non-specifically bound cells.

[0052] One or more treatment solutions or cell suspensions may then be introduced to expand the number of captured cells, activate the captured cells, deplete a subset of the captured cells, introduce exogenous genes into the captured cells, or the like. After the treatment has been completed, the vessel may then be washed again to remove the treatment solution, cellular debris, or the like and an appropriate medium introduced to maintain the viability of the bound cells. The cells may then be released by adding a medium containing interleukin-2 and a mitogenic agent, or by taking the cell capture device 26 and introducing it into an ultrasonic bath or subjecting it to mechanical vibration or vigorous pipetting. After a short period of such physical treatment or under relatively mild sonic vibration, the captured cells are released and may be harvested.

[0053] The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTALI. DEVICE AND PROCESS VALIDATION

5 A. Synthesis of N-(hydroxymethyl) 2-bromoacetamide (HMBA) and generation of the bromoacetamide polystyrene surface (BA-PS).

[0054] HMBA is synthesized by conventional means (Leonard et al., *J. Org. Chem.* **50**:2480 (1985)) from 2-bromoacetamide, available from commercial sources, in the presence of formalin at pH 10, which provides a 93% yield of the starting reactant, N-(hydroxymethyl) 2-bromoacetamide (HMBA).

[0055] The second step involves the generation of the bromoacetamide polystyrene surface (BA-PS). In this step, 2M triflic acid and 0.2M HMBA, both in tetramethylene sulfone (sulfolane), are mixed 1:1 in a volume sufficient to cover the inner surface of a polystyrene vessel being activated. The reaction is allowed to proceed at 27°C for 3 hours. The reaction solution is drained, the device washed with water, followed by ethanol, and the activated polystyrene chambers are air dried. The resulting bromoacetamide polystyrene surface is stable in room air for six (6) months.

B. Cell capture surface preparation, stabilization and sterilization.

[0056] The next step is the receptor capture (the monoclonal antibody one wishes to covalently bind to the bromoacetamide-polystyrene surface). The monoclonal antibody of interest is diluted to approximately 0.01 - 0.05 mg/ml in phosphate buffered saline, pH 7.4. The appropriate volume of diluted monoclonal antibody is introduced into the polystyrene chamber and the reaction is allowed to proceed for about two to twenty, preferably about 2 to 4 hours, at 27°C with rotation. The antibody remaining after the reaction is decanted and can be re-utilized up to 10 times in subsequent coating reactions.

[0057] The antibody bound device is then washed ten times with phosphate buffered saline (PBS), pH 7.4, and the surface is then stabilized by the addition of 2% sucrose/0.2% human serum albumin (HSA), medical grade, to each device. The sucrose/albumin solution is allowed to coat the surface, after which the excess sucrose/HSA solution is decanted and the stabilized polystyrene chambers dried 24-96 hours in a vacuum (<0.10 Torr) at 25°C. After drying, the vacuum is broken with dry nitrogen and the device is flushed with inert, dry gas and capped tightly. The device is sealed and then sterilized. Sterilization is achieved by irradiation with 2.7 ± 0.2 megarads of electron beam or gamma irradiation. Sterility tests showed that the flasks were sterile after a 14 day in situ media incubation.

C. Density of cell capture surface receptor

[0058] A variety of surface functionalization groups were employed and tested for the stability of binding of antibody to the surface. The polystyrene was functionalized using N-(hydroxymethyl)2-haloacetamide, where the halo group was chloro, bromo or iodo; diazonium and sulfonium. After monoclonal antibody attachment using these surfaces, the flasks were each washed 10 times with PBS and once with 1% SDS at 55°C for 14 hours. The plastic surface was then assayed for radioactivity of the labeled monoclonal antibodies and the results expressed as surface density for monoclonal antibody in ng/cm². The bromoacetamide has a surface density of about 250 ng/cm² of antibody, more than 2.5 times that achieved by adsorption on an Immulon-2™ (Dynatek) surface. While the bromoacetamide provided the highest surface density, the surface density for the other functionalities fell between 200 and about 240 ng/cm².

D. Stability of capture surface receptor.

[0059] The stability of the antibody binding was determined by coating the surface with 0.02 mg/ml of (³⁵S) human IgG. The flasks were washed 5 times with borate-carbonate buffer, once with borate-carbonate buffer for 8 hours and twice with borate-carbonate washes over night. Aliquots of each wash were saved and assayed for radioactivity. After the second wash, there was no evidence of any antibody leaching. In a second study, using an ELISA assay for the antibody bound to the surface, the results observed showed that the amount of extractable antibody was less than the detection limit of the assay, (7.7 ng/ml).

E. Density of cell capture by derivatized polystyrene surface

[0060] Because the receptor-derivatized polystyrene surface retains its optical clarity, the density of the captured cells per unit area of derivatized polystyrene can be calculated by direct microscopic visualization. For most cell capture applications, the density of bound cells approaches the closest packing of spheres on a monolayer. For lymphocytes of mouse or human origin, the binding density is from 0.5×10^6 cells/cm² to 1×10^6 cells/cm². Depending upon the fre-

quency and size of the target cell in the input cell suspension, the density of bound cells can vary widely, from 1×10^3 cells/cm² for large, rare target cells to greater than 10^{10} cells/cm² for small, abundant cells or particles, such as bacteria or viruses.

5 F. Specificity of cell capture by the derivatized polystyrene surface.

[0061] Cell binding to the functionalized polystyrene surface is specifically determined by the receptor bound to the polystyrene. The following experiment illustrates the specificity of cell binding. Mononuclear cells were prepared from peripheral blood by standard histopaque centrifugation and diluted to 3×10^6 cells/ml of PBS. An aliquot of the input was reserved for phenotype analysis by flow cytometry. The cell suspension was added to T-25 flasks which contained on their internal bottom surface, a purified monoclonal antibody covalently bound by the subject method with specificity for either (1) Thy 1.2 (a murine T-cell marker), (2) CD5, (3) CD8, or (4) an equimolar mixture of CD8 and CD5 antibodies (human T-cell markers). Cells were allowed to incubate for 30 min, then rocked gently and allowed to settle for an additional 30 min. Non-adherent cells (those not attached to the flask surface) were recovered by decanting and aliquots were also reserved for phenotype composition analyzed by flow cytometry. In all cases, except the Thy 1.2 flask in which no cells were seen bound to the flask, microscopic examination of the flasks showed confluent cell binding, at a density of 0.5×10^6 cells/cm². Flow cytometric analysis was performed on all input and efflux (non-adherent) cell populations for the markers CD5, CD8 (human T-cells), CD14 (human monocytes), CD16 (human NK-cells) and CD20 (human B-cells) and the relative frequencies of these markers in input and efflux compared for each flask. The results show no differences between input and efflux for the Thy 1.2 flask. The CD5 and CD8 flasks showed respectively, greater than 98% depletion of CD5 and CD8 cells in the efflux, and the CD5/8 flask showed depletion of CD5 and CD8 cells to a degree equivalent to that of either the monospecific CD5 or CD8 flask. The markers for monocytes and NK-cells and B-cells showed relative enrichment in the efflux as they were not captured by the flask. These data show that (1) cells are quantitatively and specifically captured by the cell capture device, (2) the functionalized surface does not exhibit non-specific cell binding, and (3) more than one cell phenotype can be captured simultaneously by a bi-derivatized polystyrene surface.

G. Process for cell recovery from the capture device

[0062] Two techniques were employed to recover cells from the capture device. Both show quantitative cell recovery, good viability, absence of monoclonal antibody on the surface of the recovered cells and full biological activity for both replication and function. The first method, called lymphokine release, was tested with CD8⁺ cytotoxic T-cells captured from normal human peripheral blood according to the subject method described above. After decanting of the non-adherent cells and verifying confluent binding by microscopic observation, standard tissue culture media supplemented with recombinant IL-2 (300 units/ml) (usually between 20 and 1000 units/ml) and phytohemagglutinin (PHA:Gibco 0.1 mg/ml) (usually between 0.1 and 5.0 mg/ml) were added. After 48 to 72 hours of culture, the captured CD8⁺ cells spontaneously detach from the flask, leaving all the monoclonal antibody covalently attached to the polystyrene surface.

[0063] The captured CD8 cells were shown to be free of surface-bound monoclonal antibody by flow cytometry analysis using fluoresceinated anti-mouse antibody. None of the released CD8⁺ cells were positive for surface mouse IgG. Furthermore, the flask can be re-used for cell capture by washing in PBS containing 4M MgCl which regenerates the capture surface. Such re-used flasks perform consistently for 4-6 cycles after which repeated washing reduces the bound antibody activity. Further proof of retention of the antibody by the polystyrene surface is provided by in situ polystyrene blotting studies in which radiolabeled anti-mouse antibody is reacted with the derivatized polystyrene, washed vigorously and the surface either assayed by autoradiography or by direct scintillation counting. In both sets of experiments, the polystyrene surface is fully saturated with bound monoclonal antibody indicating retention of the antibody in the device.

[0064] The detached cells, recovered by decanting, can be expanded numerically in standard tissue culture chambers supplemented with IL-2 and phytohemagglutinin. Viability by Trypan blue exclusion was shown to be greater than 98% and the recovered, homogeneous cell population could be expanded by two orders of magnitude over a period of about 10 days.

[0065] The second method for cell recovery, called ultrasonic release, utilizes an ultrasonic bath (Crest Ultrasonics model #H-4HT-1014-6) with an output of 40 to 90 kHz sonic output (main frequency at 40 kHz) evenly distributed through a water bath by means of the Crest Vibra-bar. The power supply delivers 500 watts at 40 to 90 kHz. The ultrasonic bath has an immersion tank of 10 x 14 inches, holding a volume of 6 gallons of fluid which contained one litre (0.5" from the tank bottom) for sonication in the subject studies. Immersion tanks of various sizes are commercially available. The capture device containing the bound cells is immersed in the one litre of fluid in the ultrasonic bath and the power supply and power application time experimentally determined. Depending upon the cell phenotype, times and powers varied: For example, CD4⁺ T-cells: 78% max power, 17 sec; CD8⁺ T-cells: 30% max power, 20 sec; Leu-

19 cells: 75% max power, 10 sec, etc.

[0066] To demonstrate that the cells recovered by sonication were still viable and retained their physiological activity, CD16⁺ NK-cells were recovered by sonication at maximum power for 15 to 20 seconds. The cells recovered by sonication (1) were greater than 85% viable by Trypan blue exclusion, and (2) were extremely active in a lytic assay routinely utilized to quantitate NK-cell activity. Using flow cytometric analysis, cells recovered by sonication were shown to be free of monoclonal antibody, as were cells recovered by the mitogen/lymphokine drive method described above. Thus, in cells recovered by both methods, the antibody remains behind when the cells are recovered, providing viable, homogeneous, fully functional cells free of monoclonal antibody.

10 H. Phenotypic homogeneity of released cells.

[0067] Previously (section F) analysis of input and efflux (non-captured) cell phenotypes showed that cell binding by the cell capture device is specified by the monoclonal antibody covalently bound to the device surface. In this section, data are presented to confirm and extend these findings by analyzing by flow cytometry the cells recovered from the device by lymphokine release. Mononuclear cells from peripheral blood of normal human volunteers were prepared by standard histopaque centrifugation as described and introduced at a concentration of 3×10^6 cells/ml PBS into cell capture devices containing either CD8 or CD4 monoclonal antibody covalently bound to the inner surface. After standard incubation and decanting of non-attached cells, the captured cells were recovered by incubation for 48 hours with IL-2 and PHA as described in section G. The recovered cells were then phenotyped by flow cytometry and cultured in standard culture media supplemented with IL-2 and PHA. Aliquots of cells were sampled periodically over 6-25 days in culture for flow cytometric analysis. The data show greater than 95% homogeneity for CD4⁺ and CD8⁺ surface markers on recovered cells from the CD4 and CD8 devices, respectively, at time zero (immediately after recovery from the device). More importantly, as the recovered cells logarithmically grow in culture, their phenotypic homogeneity is preserved, with cultures maintaining greater than 95% purity for CD4 and CD8 markers, respectively, over the 6-25 day culture period. Released cells are therefore homogenous in phenotype and their homogeneity is maintained during in vitro logarithmic growth.

I. Numerical expansion of released cells.

[0068] CD8⁺ cells recovered by lymphokine drive from a CD8 capture device using human peripheral blood mononuclear cells from six (6) different individuals were cultured in standard culture media supplemented with IL-2 and PHA (300 units/ml and 0.1 µg/ml, respectively) in standard culture vessels in a humidified incubator at 37°C. Cells were sampled for viability by Trypan blue exclusion and cell number by hemocytometer counting periodically over 25 days of culture. Each individual's cells were kept separated from the others. The data show greater than 95% cell viability and a two log increase in cell number over 20 days. These data demonstrate the capability of cells recovered from the capture device to exponentially expand in number in standard tissue culture.

J. Induction of proliferation of recovered cells by immobilized CD3 monoclonal antibody.

[0069] In this study, CD8⁺ cells harvested from peripheral blood of normal volunteers were captured in the subject device containing CD8 antibody and recovered by lymphokine drive. The recovered cells were then cultured in either standard tissue culture flasks using standard tissue culture medium supplemented with recombinant IL-2 and PHA, or cultured in the subject device with covalently-bound anti-CD3 monoclonal antibody using standard medium without supplementation with either recombinant IL-2 or PHA. Duplicate flasks with the anti-CD3 monoclonal antibody were employed. At time zero, equal numbers of cells were loaded, respectively, into flasks A, B and C (A=standard tissue culture flask with IL-2/PHA supplemented media; B and C=CD3 subject device without IL2 or PHA). After five days in culture, each culture was split into two aliquots and replated in identical flasks under identical culture conditions. Cells were then recounted at day 9, resulting in the following fold-expansions between days 5 and 9: A:2.7; B:2.55; C:6.75. Control cultures in which CD8⁺ cells were cultured in standard tissue culture vessels without IL-2 or PHA supplement failed to grow at all. Thus, cell expansion was achieved at the same or greater multiple using immobilized anti-CD3 antibody and the subject device as compared to IL-2/PHA supplemented media in a standard tissue culture flask. These data demonstrate that by immobilizing a T-cell activating monoclonal antibody (CD3) to the polystyrene surface according to the subject method, T-cell activation/proliferation can be achieved by the immobilized monoclonal in the absence of soluble activation factors (IL-2/PHA) in the culture medium.

II. SPECIFIC EXAMPLES

A. Bone marrow transplantation

5 [0070] In this first example, T-cell depletion for bone marrow transplantation is exemplified. Data indicate that the CD5⁺ and CD8⁺ T-cells which are present in bone marrow material cause graft-versus-host disease. A device as described above was prepared using monoclonal antibodies to CD5 and CD8 positive human T-cells. Aliquots of human bone marrow obtained from normal human volunteers were introduced into a subject device and the cells incubated as described. Non-adherent cells were recovered, phenotyped and subjected to *in vitro* cultures to quantitate enrichment for progenitor cells compared to input non-fractionated marrow. The following tables indicate typical results.

TABLE 1

| Depletion of T-cells | | | | | |
|------------------------|-----|-----|------|------|------|
| (% depletion of input) | | | | | |
| CD5 | CD8 | CD4 | CD14 | CD16 | CD19 |
| 91 | 96 | 65 | -129 | -29 | 4 |

TABLE 2

| Enrichment of Progenitors | | | | |
|---------------------------|-------|--------|-------|-------|
| (% enrichment over input) | | | | |
| CFU-EU | BFU-E | CFU-GM | CFU-M | CFU-G |
| 513 | 633 | 376 | 311 | 244 |

CFU-EU = colony forming units, erythroid units
BFU-E = burst forming units, erythroid
CFU-GM = colony forming units, granulocyte-monocyte
CFU-M = colony forming units, monocyte
CFU-G = colony forming units, granulocyte

35 [0071] The data in the tables show specific depletion of CD5⁺ and CD8⁺ cells (CD14⁺, CD16⁺ cells are enriched, CD19⁺ cells are unchanged) and 2-6-fold enrichment for progenitor cells. These data illustrate the use of the subject method to specifically deplete cells causing graft-versus-host disease while enriching for the desired progenitor cells.

[0072] In the second example of bone marrow transplantation applications, the ability of the subject device to concentrate a particular rare cell population in a mixture of cells from bone marrow or peripheral blood is demonstrated.

40 The cells to be concentrated are progenitor stem cells from human bone marrow. In this example, the subject device incorporates a CD34 monoclonal antibody covalently bound to the polystyrene surface. In the first case of this example, human bone marrow samples were introduced into the CD34 subject device, the cells incubated as described, the non-adherent cells recovered by decanting and the captured cells recovered by sonication. The three fractions, input, non-adherent and adherent cells, were assayed for CFU-C, a standard assay for progenitor cells. The following table shows the results:

TABLE 3

| | CFU-C/25,000 Cells |
|--------------------|--------------------|
| Input cells | 3 |
| Non-adherent cells | 0 |
| Adherent cells | 44 |

55 [0073] The data indicate a 15-fold increase in progenitor cells achieved by the subject device and the subject method of cell recovery by sonication.

[0074] In the second case of this example, peripheral blood mononuclear cells were introduced into another CD34 subject device. Non-adherent cells were recovered by decanting, adherent cells were again recovered by sonication. Aliquots of the input and adherent cells were analyzed for CD34⁺ phenotype. The input cells were less than 0.1%

positive for CD34⁺ cells. The adherent cells recovered by sonication were 15% CD34⁺ indicating the utility of the subject device and method for recovering viable progenitor cells from peripheral blood.

B. Anti viral cellular therapy, e.g., AIDS

5 [0075] In the next example, a process for the treatment of viral infection, e.g., AIDS, is exemplified. The technique is to expand CD8⁺ cells by capturing CD8⁺ cytotoxic T-cells from peripheral blood mononuclear cells. The captured CD8⁺ cells are then recovered by a brief culture in medium containing a lectin and recombinant IL-2 (lymphokine drive), followed by expansion of the detached cells in standard tissue culture vessels for 14 to 28 days prior to final washing and collection for reinfusion into the patient of origin.

10 [0076] Specifically, peripheral blood human mononuclear cells (PBMC) concentrated with Ficoll-Hypaque were introduced into a T-150 polystyrene flask with a anti-CD8 monoclonal antibody covalently attached. After one hour of incubation, the blood was decanted and tissue culture medium supplemented with IL-2(300 unit/ml) and PHA (0.1 µg/ml). After 48-72 hours of culture, the CD8⁺ cells spontaneously detach from the flask leaving the antibody covalently attached to the surface of the polystyrene as demonstrated by flow cytometric analysis showing the absence of monoclonal antibody on the surface of the detached cells. The detached cells are then expanded in a standard tissue culture chambers supplemented with IL-2 and PHA as above.

15 [0077] Analysis by flow cytometry showed the population to be 100% positive for CD3 and 98% positive for CD8 cell surface markers. The phenotype of the captured cells is consistent with the description reported for cytotoxic lymphocytes bearing the CD8 surface marker.

20 [0078] Captured CD8⁺ cells from six healthy donors were shown to grow logarithmically for up to 15-36 days in culture with the media containing IL-2 and PHA as above. Analysis of the cells during growth at days 7, 10, 15 and 25, show that the CD3⁺, CD8⁺ phenotype was persistent (greater than 98% positive) throughout the 25 days expansion. In a lectin-dependent cellular cytotoxicity assay using concanavalin A-coated-CEM cells, the composite lytic activity of cells from five different normal donors was determined. Substantial lysis was observed at effector-to-target ratios ranging from 2.5 to 10. These same CD8⁺ cells after expansion show no lysis of normal autologous PBMC from healthy donors. Thus, these cells have normal cytotoxic activity to appropriate target cells, while lacking autoimmune cytotoxic activity.

25 [0079] These cells were investigated to determine whether they had undergone changes which might make them susceptible to immune attack by autologous PBMCs. The results of two experiments from different donors in which the donor PMBC response to chromium-labeled self and non-self CD8 cells were examined showed that lysis occurred only for non-self CD8⁺ cells after *in vitro* priming with non-labeled, non-self CD8⁺ cells. Thus, the CD8⁺ cells do not undergo surface phenotype alterations which after reinfusion into the patient of origin, might render them targets for an autoimmune process.

30 [0080] These cells were shown to retain antigen-specific, MHC restricted cytolytic activity after isolation and expansion. CD8⁺ cells were harvested from EBV (Epstein Barr virus)-positive healthy donors and tested for specific cytotoxicity against chromium-labeled EBV-infected mitomycin C-treated autologous B-cells. During co-cultivation, reduced doses of IL-2 were added to the medium to allow for the selective expansion of CD8⁺ cells with specific reactivity against EBV-infected MHC restricted autologous B-cells. The protocol for this assay was to include a control in which CD8⁺ cells were grown but not primed and then subjected to the chromium release assay on day 9 and thereafter. The experiment included: (1) an aliquot of cells subjected to the chromium release assay on day 0 before priming, (2) another aliquot primed on day 0, primed again on day 7, and then subjected to the chromium release assay on day 9 and thereafter. The results were as follows: (1) CD8⁺ cells not exposed to EBV-transformed autologous B-cells showed no lytic activity, (2) control cultures utilizing CD8⁺ cells from EBV-sero-negative healthy donors also showed no lytic activity whether primed or not primed, (3) with primed CD8⁺ cells from EBV-seropositive donors, at effector:target ratios in the range of 3 to 12.5, percent specific lysis ranged from 25 to about 45. These results demonstrate that the CD8⁺ cells harvested and expanded after 14 days of cold culture demonstrate antigen-specific MHC restricted cellular cytotoxicity appropriate to the antigenic milieu of the host.

35 [0081] In the next study, CD8⁺ cells were obtained from HIV-positive volunteers. The cells were harvested from Ficoll-Hypaque PBMC as described above, captured with a CD8 subject device and recovered by lymphokine release as described. Logarithmic growth for 18 days in culture with nearly 100% viability was achieved with CD8⁺ cells from HIV-positive donors. The CD8⁺ phenotype was substantially retained (greater than 95% positive CD8) during *in vitro* expansion. These cells exhibited appropriate cytotoxicity against lectin coated CEM cells and exhibited no NK-like lytic activity against K562 targets. In addition, the CD8⁺ cell showed no suppressor activity in a B-cell immunoglobulin synthesis assay. The cells are not transformed, requiring constant IL-2 to remain in growth phase. The cells do not produce HIV virus and, after washing, are lymphokine, PHA and monoclonal antibody free.

40 [0082] The expanded CD8⁺ cells showed stable phenotype, normal lytic activity, maintained the absence of markers for other types of cells, and were capable of cytolytic activity against appropriate target cells. Most importantly, these CD8⁺ cells exhibited an inhibition of autologous HIV virus replication *in vitro*. This was established by combining CD4⁺

cells infected with HIV with autologous expanded CD8⁺ cells. Complete repression of HIV replication was achieved at as low a CD4:CD8 ratio as 1:0.25 after 7 days. Different time periods and different CD4:CD8 ratios were involved with different donors, but in all cases, HIV repression was complete in the autologous setting, lasting for up to 35 days in culture (the longest period tested).

[0083] In summary, these data show that CD8⁺ cells captured from PBMCs by the subject method and recovered by lymphokine drive: (1) are phenotypically pure, (2) are capable of exponential growth *in vitro*, (3) are phenotypically stable during exponential growth, (4) are capable of potent, appropriate cytotoxic activity, (5) are capable of repressing HIV replication in autologous CD4⁺ cells when the CD8⁺ cells are captured from HIV seropositive donors, (6) show, in general, MHC and antigen restricted cytotoxicity, (7) show no autoreactivity, (8) show no auto recognition, (9) show no suppressor cell activity, (10) are not transformed, (11) do not produce HIV virus, and (12) do not retain residual biologicals derived from the culture process after washing. These cells are suitable for a variety of therapeutic applications, including AIDS, cytomegalovirus infections, EBV infections, toxoplasmosis infections, etc. Furthermore, the CD8⁺ cells, when isolated by the subject method from tumors or lymphoid homogenates from cancer patients, show substantial anti-cancer activity, as shown in the next example.

C. Tumor infiltrating lymphocyte.

[0084] Cell suspensions obtained by enzymatic digestion of tumors or lymphoid tissue from cancer patients were introduced into devices containing CD4 or CD8 monoclonal antibody bound to the surface. After capturing the CD4⁺ or CD8⁺ cells and recovering them by either sonication or lymphokine drive, the recovered cells were shown to be greater than 98% viable and greater than 95% phenotypically pure CD4⁺ or CD8⁺ cells, respectively. In all cases examined, either the purified CD4⁺ or purified CD8⁺ cells exhibited at least as much autologous tumor cytotoxicity as the unseparated starting tissue suspension. The purified population did not exhibit non-specific killing of allogeneic tumor. The purified population were capable of logarithmic growth, maintaining viability, phenotypic homogeneity and autologous tumor cytotoxicity. The phenotype of the cytotoxic cell varied among tumor types, CD8⁺ predominating for melanoma and squamous cell carcinoma, CD4⁺ predominating for renal cell carcinoma.

D. Lymphokine activated killer cells (LAK).

[0085] In the next example, anti-CD5, anti-CD14 and anti-CD20 monoclonal antibodies were employed in subject devices to deplete PBMCs to enrich by negative selection for NK-cells. The antibodies were shown to be capable of depletion of the CD5⁺ phenotype by greater than about 98%, the CD14⁺ phenotype by greater than 50% and the CD20⁺ phenotype by greater than 90%. To obtain LAK cells, 1.5 X 10⁸ mononuclear cells were added to the collection device containing CD5 monoclonal antibody. After incubating for 30 min, the non-adherent cells were recovered by decanting, and transferred to a device containing covalently bound anti-CD14 antibody. After incubation, the non-adherent cells were again recovered by decanting and transferred to a third device, this one containing CD20 monoclonal antibody covalently bound to the surface. Incubation was again for 30 min at room temperature. The third population non-adherent cells were then cultured in IL-2 for 48 to 72 hours and their lytic activity assayed in standard 4-hour chromium release assays using K562 for NK activity and COLO-205 cells for LAK activity. The percent specific lysis for different effector to target ratios was determined where the effector to target ratio varied from 2.5 to 20. Using cells from several different normal donors, the enrichment of LAK activity varied from 50% to 300% over input cells cultured under identical conditions.

[0086] The cell population derived from the subject device was shown to be substantially enriched for LAK precursors, virtually free of T- and B-cells and significantly depleted of monocytes. The phenotype of the LAK precursor purified by the subject device was found to be CD3⁻, CD16⁺ and Leu 19⁺.

[0087] In the next study, the question of whether the lytic unit activity was increased out of proportion to the phenotypic enrichment of NK cells in the purified samples was addressed. The lytic units were calculated for the input and purified fractions per 10⁶ NK effector cells. A 2 to 50-fold increase in lytic units per 10⁶ NK effector cells is achieved with the three step monoclonal antibody negative depletion method described. This established that the monocyte, B-cell, T-cell depletion protocol increased by a factor of 2 to 50, the lytic unit activity expressed per NK effector cell. This increase is achieved by the removal of other cells that directly or indirectly exert inhibitory influences on LAK activity. See Nii, et al., *Int. J. Cancer* (1988) 41:33-40; Hoyer, et al., *Cancer Res.* (1986) 46:2834-2938. These authors report the down regulation by activated autologous monocytes of human lymphokine IL-2 activated killer cell activity. The subject procedure achieved a 90% reduction in total cell number resulting in the saving of culture resources required to perform the NK activation; and a 2 to 50-fold augmentation in lytic activity expressed on a per-NK effector cell basis.

[0088] The data presented above demonstrate that the subject methodology improves the efficacy of IL-2/LAK therapy, decreases the cost of the therapy, and reduces the toxicity of the procedure by lowering the total number of cells obtained by leukapheresis necessary to generate the targeted total lytic activity for re-infusion after IL-2 activation.

E. Suppressor-inducer cells.

[0089] In a similar manner to the previous procedures, the monoclonal antibody 2H4 which binds to the suppressor-inducer cell, a cell which induces specific suppressor cells, can be utilized to harvest, recover, activate and expand suppressor-inducer cells to treat autoimmune disease. The suppressor-inducer cell would be positively selected from PBMCs, recovered from the collection device by sonication, expanded and activated numerically according to the prior procedures. These expanded and activated suppressor-inducer cells could then be reinfused to the patient of origin with the autoimmune disease in question, which would result in the induction of suppressor cell activity appropriate to the patient's pathophysiology.

[0090] The above results demonstrate the power of the subject device and process in isolating a wide variety of cells with different surface markers. The procedures may be used in research, diagnosis and therapy. Furthermore, the procedure allows for collection, expansion and activation of cells while retaining a very high percentage of viability of the cells. In addition, antigenic components of blood or tissue may be taken from a patient, such as immune complexes or tumor cells or normal tissue and used to activate or deactivate specific responses to an antigen or cell. Thus, cellular responses may be provided to a wide variety of diseases, including: genetic diseases, where stem cells may be transfected so as to modify their phenotype; autoimmune diseases, where suppressor cells may be used to suppress an immune response; cancer and viral diseases where killer cells may be used in their treatment; and pathogen derived diseases where helper and B-cells may be used in protection against a wide variety of pathogens.

[0091] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[0092] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto.

Claims

1. A method of changing the cellular composition of a mixture of cells, the method comprising passing the fluid through a cell capture device so that the cells contact at least one internal flat polystyrene surface of the device which is a sheet, fibre, container wall or partition the surface having covalently bound at least one receptor specific for at least one ligand present on at least some of the cells in the fluid for sufficient time for the cells containing said ligand to specifically bind to the surface; and
removing cells which are not specifically bound without significantly disturbing specifically bound cells.
2. A method according to claim 1 wherein the fibre is a hollow fibre and/or the composition comprises a physiological fluid.
3. A method according to claim 1 or 2 further comprising, after the removing step, releasing the bound cells, substantially free of the receptor, so as to obtain a composition of specifically bound cells.
4. A method according to claim 3 wherein releasing is by a physical method or comprises treatment with an interleukin, growth factor and/or a mitogenic agent.
5. A method according to any preceding claim wherein the cells are hematopoietic cells.
6. A method according to any preceding claim further comprising contacting the specifically bound cells, optionally free of cells which are not specifically bound, with at least one of an activating agent, an antigen, a cell capable of binding to a surface protein of the bound cell, an immune complex, a mitogenic agent, a transfection vector, an activating antibody, or a cytotoxic agent.
7. A method according to claim 1 or 2 for use in the preparation of a therapeutic cellular composition for treating a patient, wherein the receptors comprise monoclonal antibodies.
8. A method according to claim 6 further comprising expanding the population of the specifically bound cells.
9. A method of obtaining a therapeutic cellular composition comprising specifically bound cells being substantially free of receptor, the method comprising a method according to claim 1 and releasing cells, that have been bound

to the surface which contains covalently bound receptors specific for at least one ligand present on the specifically bound cells, from that surface.

5 10. A method according to claim 9 wherein the release is by sonication or comprises treatment with an interleukin, growth factor and/or a mitogenic agent.

11. A method according to claim 9 further comprising expanding the population of the specifically bound cells.

10 12. A method according to claim 9 or 10 wherein the receptors comprise monoclonal antibodies.

13. A method according to claim 7 or 12 wherein the therapeutic cellular composition is suitable for use in bone marrow transplantation,

15 the mixture of cells comprises bone marrow cells from a bone marrow donor; and the monoclonal antibodies are specific for CD8 and/or CD5.

14. A method according to claim 12 wherein the antibodies are specific for CD8 and CD5.

15 20 15. A method according to claim 7 or 12 wherein the therapeutic cellular composition is suitable for use in transplantation,

the mixture of cells comprises peripheral blood or bone marrow; and the monoclonal antibodies are specific for CD34.

25 16. A method according to claim 7 or 12 wherein the therapeutic cellular composition is suitable for use in gene therapy,

the mixture of cells comprises peripheral blood or bone marrow; and the monoclonal antibodies are specific for CD34.

30 17. A method according to claim 7 or 12 wherein the therapeutic cellular composition is suitable for treating an individual infected with a virus;

the mixture of cells is a composition of peripheral blood mononuclear cells; and the monoclonal antibodies are specific for CD8.

35 18. A method according to claim 7 or 12 wherein the therapeutic cellular composition is suitable for treating a patient with a neoplasm,

40 the mixture of cells comprises a tumor or lymphoid cell or tissue suspension from a patient with a neoplasm; and the monoclonal antibodies are specific for CD8 or CD4.

19. A method according to claim 7 or 12 wherein the therapeutic cellular composition comprises lymphokine activated killer cells,

45 the mixture of cells comprises peripheral blood mononuclear cells; and the monoclonal antibodies are specific for CD5, CD14 or CD20.

20. A method according to claim 7 or 12 wherein the therapeutic cellular composition is suitable for treating an autoimmune disease,

50 the mixture of cells comprises peripheral blood mononuclear cells; and the monoclonal antibodies are specific for suppressor-inducer cells.

55 **Patentansprüche**

1. Verfahren zum Ändern der zellulären Zusammensetzung eines Zellgemischs, welches Verfahren das Durchleiten der Flüssigkeit durch eine Zelleinfangvorrichtung in derartiger Weise umfaßt, daß die Zellen mindestens eine in-

terne flache Polystyrol-Oberfläche der Vorrichtung, bei der es sich um eine Platte, Faser, Behälterwand oder Zwischenwand handelt, kontaktieren, wobei die Oberfläche an mindestens einen Rezeptor kovalent gebunden hat, der für mindestens einen Liganden spezifisch ist, der auf zumindest einem Teil der Zellen in der Flüssigkeit vorhanden ist, über einen ausreichend langen Zeitraum hinweg, daß Zellen, die diesen Liganden enthalten, spezifisch an die Oberfläche binden; und

Entfernen der Zellen, die nicht spezifisch gebunden sind, ohne im wesentlichen spezifisch gebundene Zellen zu zerstören.

2. Verfahren nach Anspruch 1, wobei die Faser eine Hohlfaser ist und/oder die Zusammensetzung eine physiologische Flüssigkeit umfaßt.

3. Verfahren nach Anspruch 1 oder 2, außerdem umfassend, nach dem Entfernungsschritt, die Freisetzung der gebundenen Zellen, im wesentlichen in Rezeptor-freier Form, um eine Zusammensetzung der spezifisch gebundenen Zellen zu erhalten.

4. Verfahren nach Anspruch 3, wobei die Freisetzung mittels eines physikalischen Verfahrens oder durch Behandeln mit einem Interleukin, Wachstumsfaktor und/oder einem Mitogen erfolgt.

5. Verfahren nach einem der vorangehenden Ansprüche, wobei die Zellen hämopoetische Zellen sind.

6. Verfahren nach einem der vorangehenden Ansprüche, außerdem umfassend das Zusammenbringen der spezifisch gebundenen Zellen, wahlweise frei von den nicht spezifisch gebundenen Zellen, mit mindestens einem von einem Aktivierungsmittel, einem Antigen, einer zum Binden an ein Oberflächenprotein der gebundenen Zelle fähigen Zelle, einem Immunkomplex, einem Mitogen, einem Transfektionsvektor, einem aktivierenden Antikörper oder einem zytotoxischen Mittel.

7. Verfahren nach Anspruch 1 oder 2 zur Verwendung bei der Herstellung einer therapeutischen zellulären Zusammensetzung zur Behandlung von Patienten, wobei die Rezeptoren monoklonale Antikörper umfassen.

8. Verfahren nach Anspruch 6, außerdem umfassend das Erweitern der Population der spezifisch gebundenen Zellen.

9. Verfahren zum Erhalt einer therapeutischen zellulären Zusammensetzung, umfassend spezifisch gebundene Zellen, die im wesentlichen frei von Rezeptor sind, wobei das Verfahren ein Verfahren nach Anspruch 1 und das Freisetzen von solchen Zellen, die an die Oberfläche gebunden haben, welche kovalent gebundene Rezeptoren enthält, die für mindestens einen auf den spezifisch gebundenen Zellen vorhandenen Liganden spezifisch sind, von dieser Oberfläche umfaßt.

10. Verfahren nach Anspruch 9, wobei die Freisetzung durch Beschallung erfolgt oder das Behandeln mit einem Interleukin, Wachstumsfaktor und/oder einem Mitogen umfaßt.

11. Verfahren nach Anspruch 9, außerdem umfassend das Erweitern der Population der spezifisch gebundenen Zellen.

12. Verfahren nach Anspruch 9 oder 10, wobei die Rezeptoren monoklonale Antikörper umfassen.

13. Verfahren nach Anspruch 7 oder 12, wobei die therapeutische zelluläre Zusammensetzung zur Verwendung bei Knochenmarktransplantationen geeignet ist,

das Zellgerüst Knochenmarkzellen eines Knochenmarkspenders umfaßt; und

die monoklonalen Antikörper für CD8 und/oder CD5 spezifisch sind.

14. Verfahren nach Anspruch 12, wobei die Antikörper für CD8 und CD5 spezifisch sind.

15. Verfahren nach Anspruch 7 oder 12, wobei die therapeutische zelluläre Zusammensetzung zur Verwendung bei Transplantationen geeignet ist,

das Zellgemisch peripheres Blut oder Knochenmark umfaßt; und

die monoklonalen Antikörper für CD34 spezifisch sind.

- 5 16. Verfahren nach Anspruch 7 oder 12, wobei die therapeutische zelluläre Zusammensetzung zur Verwendung in der Gentherapie geeignet ist,

das Zellgemisch peripheres Blut oder Knochenmark umfaßt; und

10 die monoklonalen Antikörper für CD34 spezifisch sind.

- 15 17. Verfahren nach Anspruch 7 oder 12, wobei die therapeutische zelluläre Zusammensetzung zur Behandlung eines mit einem Virus infizierten Patienten geeignet ist,

das Zellgemisch eine Zusammensetzung aus mononuklearen Zellen des peripheren Blutes ist; und

die monoklonalen Antikörper für CD8 spezifisch sind.

- 20 18. Verfahren nach Anspruch 7 oder 12, wobei die therapeutische zelluläre Zusammensetzung zur Behandlung eines Patienten mit einem Neoplasma geeignet ist,

das Zellgemisch eine Tumor- oder lymphoide Zell- oder Gewebe-Suspension von einem Patienten mit einem Neoplasma umfaßt; und

25 die monoklonalen Antikörper für CD8 oder CD4 spezifisch sind.

19. Verfahren nach Anspruch 7 oder 12, wobei die therapeutische zelluläre Zusammensetzung Lymphokin-aktivierte Killerzellen umfaßt,

30 das Zellgemisch monoklonale Zellen des peripheren Blutes umfaßt; und

die monoklonalen Antikörper für CD5, CD14 oder CD20 spezifisch sind.

- 35 20. Verfahren nach Anspruch 7 oder 12, wobei die therapeutische zelluläre Zusammensetzung zur Behandlung einer Autoimmunerkrankung geeignet ist,

das Zellgemisch monoklonale Zellen des peripheren Blutes umfaßt; und

40 die monoklonalen Antikörper für Suppressor-induzierte Zellen spezifisch sind.

Revendications

- 45 1. Méthode destinée à changer la composition cellulaire d'un mélange de cellules, la méthode comprenant le passage du liquide à travers un dispositif de capture des cellules tel que les cellules entrent en contact avec au moins une surface interne plate en polystyrène du dispositif qui est une feuille, fibre, paroi ou cloison d'un récipient, la surface présentant, lié de manière covalente, au moins un récepteur spécifique d'au moins un ligand présent sur au moins certaines des cellules dans le liquide pendant un temps suffisant pour que les cellules possédant ledit ligand se lient de manière spécifique à la surface ; et

50 à éliminer des cellules qui ne sont pas liées de manière spécifique sans perturber significativement les cellules liées de manière spécifique.

- 55 2. Méthode selon la revendication 1 dans laquelle la fibre est une fibre creuse et/ou la composition comprend un liquide physiologique.

3. Méthode selon la revendication 1 ou 2 comprenant en outre, après l'étape d'élimination, la libération des cellules liées, substantiellement dépourvues du récepteur, de façon à obtenir une composition de cellules liées de manière spécifique.

4. Méthode selon la revendication 3 dans laquelle la libération est réalisée par une méthode physique ou comprend un traitement avec une interleukine, un facteur de croissance et/ou un agent mitogène.
5. Méthode selon l'une quelconque des revendications précédentes dans laquelle les cellules sont des cellules hématopoïétiques.
6. Méthode selon l'une quelconque des revendications précédentes comprenant en outre la mise en contact des cellules liées de manière spécifique, éventuellement dépourvues de cellules qui ne sont pas liées de manière spécifique, avec au moins un élément parmi un agent activant, un antigène, une cellule capable de se lier à une protéine de surface de la cellule liée, un complexe immun, un agent mitogène, un vecteur de transfection, un anticorps activateur ou un agent cytotoxique.
7. Méthode selon la revendication 1 ou 2 pour une utilisation dans la préparation d'une composition cellulaire thérapeutique destinée au traitement d'un patient, dans laquelle les récepteurs comprennent des anticorps monoclonaux.
8. Méthode selon la revendication 6 comprenant en outre l'amplification de la population de cellules liées de manière spécifique.
9. Méthode d'obtention d'une composition cellulaire thérapeutique comprenant des cellules liées de manière spécifique qui sont substantiellement dépourvues de récepteur, la méthode comprenant une méthode selon la revendication 1 et les cellules libérées, qui ont été liées à la surface qui contient, liés de manière covalente, des récepteurs spécifiques d'au moins un ligand présent sur les cellules liées de manière spécifique, à partir de cette surface.
10. Méthode selon la revendication 9 dans laquelle la libération est réalisée par sonification ou comprend un traitement avec une interleukine, un facteur de croissance et/ou un agent mitogène.
11. Méthode selon la revendication 9 comprenant en outre l'amplification de la population des cellules liées de manière spécifique.
12. Méthode selon la revendication 9 ou 10 dans laquelle les récepteurs comprennent des anticorps monoclonaux.
13. Méthode selon la revendication 7 ou 12 dans laquelle la composition cellulaire thérapeutique convient pour une utilisation dans la transplantation de moelle osseuse,
le mélange de cellules comprend des cellules de moelle osseuse d'un donneur de moelle osseuse ; et
les anticorps monoclonaux sont spécifiques de CD8 et/ou CD5.
14. Méthode selon la revendication 12 dans laquelle les anticorps sont spécifiques de CD8 et CD5.
15. Méthode selon la revendication 7 ou 12 dans laquelle la composition cellulaire thérapeutique convient pour une utilisation dans la transplantation,
le mélange de cellules comprend du sang périphérique ou de la moelle osseuse ; et
les anticorps monoclonaux sont spécifiques de CD34.
16. Méthode selon la revendication 7 ou 12 dans laquelle la composition cellulaire thérapeutique convient pour une utilisation en thérapie génique,
le mélange de cellules comprend du sang périphérique ou de la moelle osseuse ; et
les anticorps monoclonaux sont spécifiques de CD34.
17. Méthode selon la revendication 7 ou 12 dans laquelle la composition cellulaire thérapeutique convient pour traiter un individu infecté par un virus ;

le mélange de cellules est une composition de cellules mononucléaires de sang périphérique ; et
les anticorps monoclonaux sont spécifiques de CD8.

- 5 18. Méthode selon la revendication 7 ou 12 dans laquelle la composition cellulaire thérapeutique convient pour le traitement d'un patient souffrant d'un néoplasme,

le mélange de cellules comprend une tumeur ou une suspension de tissu ou de cellules lymphoïdes d'un patient souffrant d'un néoplasme ; et

10 les anticorps monoclonaux sont spécifiques de CD8 ou CD4.

- 15 19. Méthode selon la revendication 7 ou 12 dans laquelle la composition cellulaire thérapeutique comprend des cellules tueuses activées par une lymphokine,

le mélange de cellules comprend des cellules mononucléaires de sang périphérique ; et

les anticorps monoclonaux sont spécifiques de CD5, CD14 ou CD20.

- 20 20. Méthode selon la revendication 7 ou 12 dans laquelle la composition cellulaire thérapeutique convient pour le traitement d'une maladie autoimmune,

le mélange de cellules comprend des cellules mononucléaires de sang périphérique ; et

25 les anticorps monoclonaux sont spécifiques de cellules suppresseurs-inductrices.

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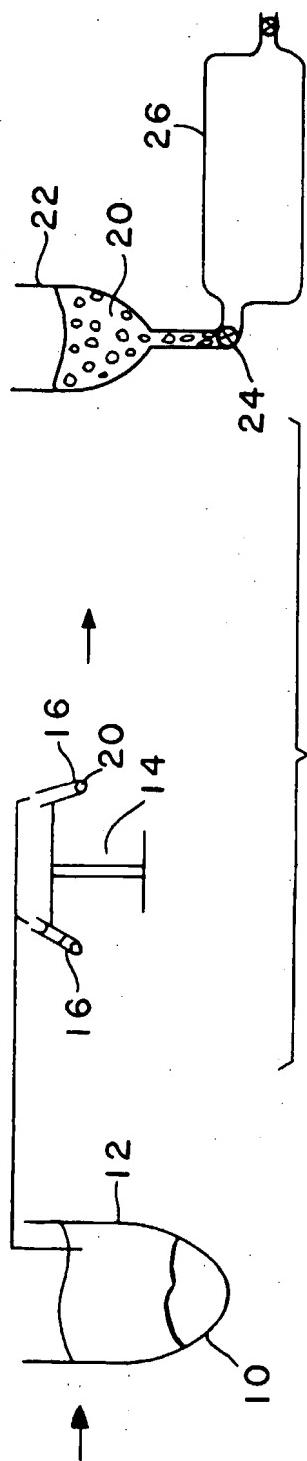
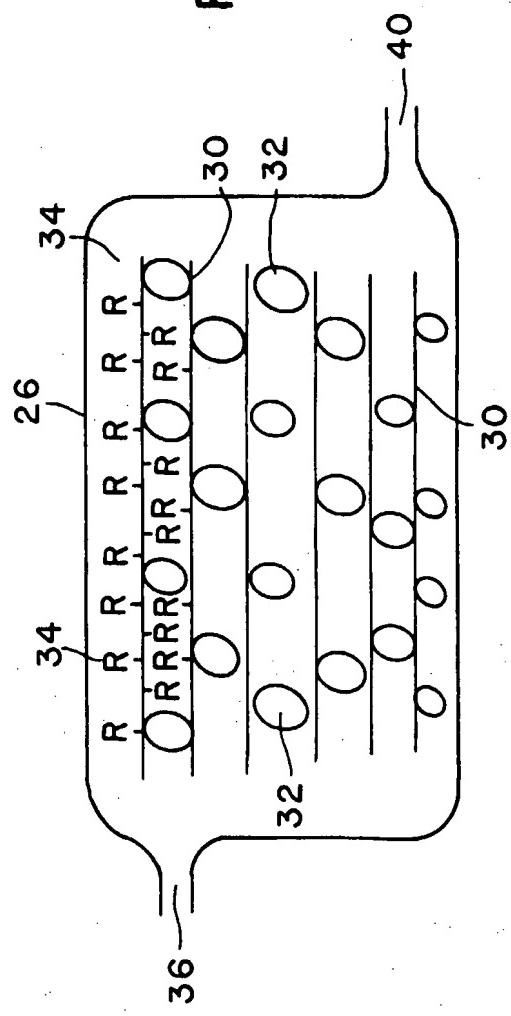


FIG. 2



(19)



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Description**FIELD OF THE INVENTION**

5 The invention relates generally to the field of cytogenetics, and more particularly, to the field of molecular cytogenetics. The invention concerns methods for identifying and classifying chromosomes. Still more particularly, this invention concerns the use of nucleic acid probes which can be designed by the processes described herein to produce staining distributions that can extend along one or more whole chromosomes, and/or along a region or regions on one or more chromosomes, including staining patterns that extend over the whole genome. Staining patterns can be tailored
10 for any desired cytogenetic application, including prenatal, tumor and disease related cytogenetic applications, among others. The invention provides for methods of staining chromosomes with compositions of nucleic acid probes to identify normal chromosomes and chromosomal abnormalities in interphase nuclei. The probe-produced staining patterns of this invention facilitate the microscopic and/or flow cytometric identification of normal and abnormal chromosomes and the characterization of the genetic nature of particular abnormalities.

15 Although most of the examples herein concern human chromosomes and much of the language herein is directed to human concerns, the concept of using nucleic acid probes for staining or painting chromosomes is applicable to chromosomes from any source including both plants and animals.

BACKGROUND OF THE INVENTION

20 Chromosome abnormalities are associated with genetic disorders, degenerative diseases, and exposure to agents known to cause degenerative diseases, particularly cancer, German, "Studying Human Chromosomes Today," American Scientist, Vol. 58, pp. 182-201 (1970); Yunis, "The Chromosomal Basis of Human Neoplasia," Science, Vol. 221, pp. 227-236 (1983); and German, "Clinical Implication of Chromosome Breakage," in Genetic Damage in Man Caused by Environmental Agents, Berg, Ed., pp. 65-86 (Academic Press, New York, 1979). Chromosomal abnormalities can be of several types, including: extra or missing individual chromosomes, extra or missing portions of a chromosome (segmental duplications or deletions), breaks, rings and chromosomal rearrangements, among others.
25 Chromosomal or genetic rearrangements include translocations (transfer of a piece from one chromosome onto another chromosome), dicentrics (chromosomes with two centromeres), inversions (reversal in polarity of a chromosomal segment), insertions, amplifications, and deletions.

30 Detectable chromosomal abnormalities occur with a frequency of one in every 250 human births. Abnormalities that involve deletions or additions of chromosomal material alter the gene balance of an organism and generally lead to fetal death or to serious mental and physical defects. Down syndrome can be caused by having three copies of chromosome 21 instead of the normal 2. This syndrome is an example of a condition caused by abnormal chromosome number, or aneuploidy. Down syndrome can also be caused by a segmental duplication of a subregion on chromosome 21 (such as, 21q22), which can be present on chromosome 21 or on another chromosome. Edward syndrome (18+), Patau syndrome (13+), Turner syndrome (XO) and Klinefelter syndrome (XXY) are among the most common numerical aberrations. [Epstein, The Consequences of Chromosome Imbalance: Principles, Mechanisms and Models (Cambridge Univ. Press 1986); Jacobs, Am. J. Epidemiol., 105: 180 (1977); and Lubs et al., Science, 169: 495 (1970).]

35 Retinoblastoma (del 13q14), Prader-Willi syndrome (del 15q11- q13), Wilm's tumor (del 11p13) and Cri-du-chat syndrome (del 5p) are examples of important disease linked structural aberrations. [Nora and Fraser, Medical Genetics: Principles and Practice, (Lea and Febiger 1989).]

40 Measures of the frequency of structurally aberrant chromosomes, for example, dicentric chromosomes, caused by clastogenic agents, such as, ionizing radiation or chemical mutagens, are widely used as quantitative indicators of genetic damage caused by such agents, Biochemical Indicators of Radiation Injury in Man (International Atomic Energy Agency, Vienna, 1971); and Berg, Ed. Genetic Damage in Man Caused by Environmental Agents (Academic Press, New York, 1979). A host of potentially carcinogenic and teratogenic chemicals are widely distributed in the environment because of industrial and agricultural activity. These chemicals include pesticides, and a range of industrial wastes and by-products, such as halogenated hydrocarbons, vinyl chloride, benzene, arsenic, and the like, Kraybill et al., Eds., Environmental Cancer (Hemisphere Publishing Corporation, New York, 1977). Sensitive measures of chromosomal breaks and other abnormalities could form the basis of improved dosimetric and risk assessment methodologies for evaluating the consequences of exposure to such occupational and environmental agents.

45 Current procedures for genetic screening and biological dosimetry involve the analysis of karyotypes. A karyotype is the particular chromosome complement of an individual or of a related group of individuals, as defined both by the number and morphology of the chromosomes usually in mitotic metaphase. It includes such things as total chromosome number, copy number of individual chromosome types (e.g., the number of copies of chromosome X), and chromosomal morphology, e.g., as measured by length, centromeric index, connectedness, or the like. Chromosomal abnormalities can be detected by examination of karyotypes. Karyotypes are conventionally determined by staining an organism's

metaphase, or otherwise condensed (for example, by premature chromosome condensation) chromosomes. Condensed chromosomes are used because, until recently, it has not been possible to visualize interphase chromosomes due to their dispersed condition and the lack of visible boundaries between them in the cell nucleus.

A number of cytological techniques based upon chemical stains have been developed which produce longitudinal patterns on condensed chromosomes, generally referred to as bands. The banding pattern of each chromosome within an organism usually permits unambiguous identification of each chromosome type, Latt, "Optical Studies of Metaphase Chromosome Organization," Annual Review of Biophysics and Bioengineering, Vol. 5, pp. 1-37 (1976). Accurate detection of some important chromosomal abnormalities, such as translocations and inversions, has required such banding analysis.

Unfortunately, such conventional banding analysis requires cell culturing and preparation of high quality metaphase spreads, which is time consuming and labor intensive, and frequently difficult or impossible. For example, cells from many tumor types are difficult to culture, and it is not clear that the cultured cells are representative of the original tumor cell population. Fetal cells capable of being cultured need to be obtained by invasive means and need to be cultured for several weeks to obtain enough metaphase cells for analysis. In many cases, the banding patterns on the abnormal chromosomes do not permit unambiguous identification of the portions of the normal chromosomes that make them up. Such identification may be important to indicate the location of important genes involved in the abnormality. Further, the sensitivity and resolving power of current methods of karyotyping are limited by the fact that multiple chromosomes or chromosomal regions have highly similar staining characteristics, and that abnormalities (such as deletions) which involve only a fraction of a band are not detectable. Therefore, such methods are substantially limited for the diagnosis and detailed analysis of contiguous gene syndromes, such as partial trisomy, Prader-Willi syndrome [Emanuel, Am. J. Hum. Genet., 43: 575 (1988); Schmickel, J. Pediatr., 109: 231 (1986)] and retinoblastoma [Sparkes, Biochem. Biophys. Acta., 780: 95 (1985)].

Thus, conventional banding analysis has several important limitations, which include the following. 1) It is labor intensive, time consuming, and requires a highly trained analyst. 2) It can be applied only to condensed chromosomes. 3) It does not allow for the detection of structural aberrations involving less than 3-15 megabases (Mb), depending upon the nature of the aberration and the resolution of the banding technique [Landegren et al., Science, 242: 229 (1988)]. This invention provides for probe compositions and methods to overcome such limitations of conventional banding analysis.

The chemical staining procedures of the prior art provide patterns over a genome for reasons not well understood and which cannot be modified as required for use in different applications. Such chemical staining patterns were used to map the binding site of probes. However, only occasionally, and with great effort, was *in situ* hybridization used to obtain some information about the position of a lesion, for example, a breakpoint relative to a particular DNA sequence. The present invention overcomes the inflexibility of chemical staining in that it stains a genome in a pattern based upon nucleic acid sequence; therefore the pattern can be altered as required by changing the nucleic acid sequence of the probe. The probe-produced staining patterns of this invention provide reliable fundamental landmarks which are useful in cytogenetic analysis.

Automated detection of structural abnormalities of chromosomes with image analysis of chemically stained bands would require the development of a system that can detect and interpret the banding patterns produced on metaphase chromosomes by conventional techniques. It has proven to be very difficult to identify reliably by automated means normal chromosomes that have been chemically stained; it is much more difficult to differentiate abnormal chromosomes having structural abnormalities, such as, translocations. Effective automated detection of translocations in conventionally banded chromosomes has not been accomplished after over a decade of intensive work. The probe-produced banding patterns of this invention are suitable for such automated detection and analysis.

In recent years rapid advances have taken place in the study of chromosome structure and its relation to genetic content and DNA composition. In part, the progress has come in the form of improved methods of gene mapping based on the availability of large quantities of pure DNA and RNA fragments for probes produced by genetic engineering techniques, e.g., Kao, "Somatic Cell Genetics and Gene Mapping," International Review of Cytology, Vol. 85, pp. 109-146 (1983), and D'Eustachio et al., "Somatic Cell Genetics in Gene Families," Science, Vol. 220, pp. 9, 19-924 (1983). The probes for gene mapping comprise labeled fragments of single-stranded or double-stranded DNA or RNA which are hybridized to complementary sites on chromosomal DNA. With such probes it has been crucially important to produce pure, or homogeneous, probes to minimize hybridizations at locations other than at the site of interest, - Henderson, "Cytological Hybridization to-Mammalian-Chromosomes," International Review of Cytology, Vol. 76, pp. 1-46 (1982).

The hybridization process involves unravelling, or melting, the double-stranded nucleic acids of the probe and target by heating, or other means (unless the probe and target are single-stranded nucleic acids). This step is sometimes referred to as denaturing the nucleic acid. When the mixture of probe and target nucleic acids cool, strands having complementary bases recombine, or anneal. When a probe anneals with a target nucleic acid, the probe's location on the target can be detected by a label carried by the probe or by some intrinsic characteristics of the probe

or probe-target duplex. When the target nucleic acid remains in its natural biological setting, e.g., DNA in chromosomes, mRNA in cytoplasm, portions of chromosomes or cell nuclei (albeit fixed or altered by preparative techniques), the hybridization process is referred to as *in situ* hybridization.

In *situ* hybridization probes were initially limited to identifying the location of genes or other well defined nucleic acid sequences on chromosomes or in cells. Comparisons of the mapping of single-copy probes to normal and abnormal chromosomes were used to examine chromosomal abnormalities. Cannizzaro et al., Cytogenetics and Cell Genetics, 39:173-178 (1985). Distribution of the multiple binding sites of repetitive probes could also be determined.

Hybridization with probes which have one target site in a haploid genome, single-copy or unique sequence probes, has been used to map the locations of particular genes in the genome [Harper and Saunders, "Localization of the Human Insulin Gene to the Distal End of the Short Arm of Chromosome 11," Proc. Natl. Acad. Sci., Vol. 78, pp. 4458-4460 (1981); Kao et al., "Assignment of the Structural Gene Coding for Albumin to Chromosome 4," Human Genetics, Vol. 62, pp. 337-341 (1982)]; but such hybridizations are not reliable when the size of the target site is small. As the amount of target sequence for low complexity single-copy probes is small, only a portion of the potential target sites in a population of cells form hybrids with the probe. Therefore, mapping the location of the specific binding site of the probe has been complicated by background signals produced by non-specific binding of the probe and also by noise in the detection system (for example, autoradiography or immunochemistry). The unreliability of signals for such prior art single-copy probes has required statistical analysis of the positions of apparent hybridization signals in multiple cells to map the specific binding site of the probe.

Different repetitive sequences may have different distributions on chromosomes. They may be spread over all chromosomes as in the just cited reference, or they may be concentrated in compact regions of the genome, such as, on the centromeres of the chromosomes, or they may have other distributions. In some cases, such a repetitive sequence is predominantly located on a single chromosome, and therefore is a chromosome-specific repetitive sequence. [Willard et al., "Isolation and Characterization of a Major Tandem Repeat Family from the Human X Chromosome," Nucleic Acids Research, Vol. 11, pp. 2017-2033 (1983).]

A probe for repetitive sequences shared by all chromosomes can be used to discriminate between chromosomes of different species if the sequence is specific to one of the species. Total genomic DNA from one species which is rich in such repetitive sequences can be used in this manner. [Pinkel et al. (III), PNAS USA, 83: 2934 (1986); Manuelidis, Hum. Genet., 71: 288 (1985) and Durnam et al., Somatic Cell Molec. Genet., 11: 571 (1985).]

Recently, there has been an increased availability of probes for repeated sequences (repetitive probes) that hybridize intensely and specifically to selected chromosomes. [Trask et al., Hum. Genet., 78: 251 (1988) and references cited therein.] Such probes are now available for over half of the human chromosomes. In general, they bind to repeated sequences on compact regions of the target chromosome near the centromere. However, one probe has been reported that hybridizes to human chromosome 1p36, and there are several probes that hybridize to human chromosome Yq. Hybridization with such probes permits rapid identification of chromosomes in metaphase spreads, determination of the number of copies of selected chromosomes in interphase nuclei [Pinkel et al. (I), PNAS USA, 83:2934 (1986); Pinkel et al. (II), Cold Spring Harbor Symp. Quant. Biol., 51:151 (1986) and Cremer et al., Hum. Genet., 74:346 (1986)] and determination of the relative positions of chromosomes in interphase nuclei [Trask et al., supra; Pinkel et al. (I), supra; Pinkel et al. (II), supra; Manuelidis, PNAS USA, 81:3123 (1984); Rappold et al., Hum. Genet., 67:317 (1984); Schardin et al., Hum. Genet., 71:282 (1985); and Manuelidis, Hum. Genet., 71:288 (1985)].

Lichter et al., Proc. Natl. Acad. Sci. USA, 85:9664 (1988) disclose the detection of human chromosome 21 aberrations by *in situ* hybridization in both metaphase and interphase cells. Cremer et al., Hum. Genet., 80 : 235 (1988) similarly detected chromosomal aberrations using biotinylated DNA library probes.

However, many applications are still limited by the lack of appropriate probes. For example, until the methods described herein were invented, probes with sufficient specificity for prenatal diagnosis were not available for chromosome 13. Further, repetitive probes are not very useful for detection of structural aberrations since the probability is low that the aberrations will involve the region to which the probe hybridizes.

This invention overcomes the prior art limitations on the use of probes and dramatically enhances the application of *in situ* hybridization for cytogenetic analysis. As indicated above, prior art probes have not been useful for in-depth cytogenetic analysis. Low complexity single-copy probes do not at this stage of hybridization technology generate reliable signals. Although repetitive probes do provide reliable signals, such signals cannot be tailored for different applications because of the fixed distribution of repetitive sequences in a genome. The probes of this invention combine the hybridization reliability of repetitive probes with the flexibility of being able to tailor the binding pattern of the probe to any desired application.

The enhanced capabilities of the probes of this invention come from their increased complexity. Increasing the complexity of a probe increases the probability, and therefore the intensity, of hybridization to the target region, but also increases the probability of non-specific hybridizations resulting in background signals. However, within the concept of this invention, it was considered that such background signals would be distributed approximately randomly over the genome. Therefore, the net result is that the target region could be visualized with increased contrast against

such background signals.

Exemplified herein are probes in an approximate complexity range of from about 50,000 bases (50 kb) to hundreds of millions of bases. Such representative probes are for compact loci and whole human chromosomes. Prior to this invention, probes employed for *in situ* hybridization techniques had complexities below 40 kb, and more typically on the order of a few kb.

Staining chromosomal material with the probes of this invention is significantly different from the chemical staining of the prior art. The specificity of the probe produced staining of this invention arises from an entirely new source - the nucleic acid sequences in a genome. Thus, staining patterns of this invention can be designed to highlight fundamental genetic information important to particular applications.

The procedures of this invention to construct probes of any desired specificity provide significant advances in a broad spectrum of cytogenetic studies. The analysis can be carried out on interphase nuclei. The techniques of this invention can be especially advantageous for applications where high-quality banding by conventional methods is difficult or suspected of yielding biased information, e.g., in tumor cytogenetics. Reagents targeted to sites of lesions known to be diagnostically or prognostically important, such as tumor type-specific translocations, permit rapid recognition of such abnormalities. Where speed of analysis is the predominant concern, e.g., detection of low-frequency chromosomal aberrations induced by toxic environmental agents, the compositions of this invention permit a dramatic increase in detection efficiency in comparison to previous techniques based on conventional chromosome banding.

Further, prenatal screening for disease-linked chromosome aberrations is enhanced by the rapid detection of such aberrations by the methods and compositions of this invention. Interphase aneuploidy analysis according to this invention is particularly significant for prenatal diagnosis in that it yields more rapid results than are available by cell culture methods. Further, fetal cells separated from maternal blood, which cannot be cultured by routine procedures and therefore cannot be analysed by conventional karyotyping techniques, can be examined by the methods and compositions of this invention. In addition, the intensity, contrast and color combinations of the staining patterns, coupled with the ability to tailor the patterns for particular applications, enhance the opportunities for automated cytogenetic analysis, for example, by flow cytometry or computerized microscopy and image analysis.

This application specifically describes chromosome specific reagents for the detection of genetic translocations and methods of using such reagents to detect such translocations. Representative genetic translocations so detected are those that produce a fusion gene - BCR-ABL - that is diagnostic for chronic myelogenous leukemia (CML).

Chronic myelogenous leukemia (CML) is a neoplastic proliferation of bone marrow cells genetically characterized by the fusion of the BCR and ABL genes on chromosomes 9 and 22. That fusion usually involves a reciprocal translocation t(9;22)(q34;q11), which produces the cytogenetically distinctive Philadelphia chromosome (Ph¹). However, more complex rearrangements may cause BCR-ABL fusion. At the molecular level, fusion can be detected by Southern analysis or by *in vitro* amplification of the mRNA from the fusion gene using the polymerase chain reaction (PCR). Those techniques are sensitive but cannot be applied to single cells.

Clearly, a sensitive method for detecting chromosomal abnormalities and, more specifically, genetic rearrangements, such as, for example, the tumor specific arrangements associated with CML, would be a highly useful tool for genetic screening. This invention provides such tools.

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Fusion of the proto-oncogene c-ABL from the long arm of chromosome 9 with the BCR gene of chromosome 22

20 is a consistent finding in CML (1-3). That genetic change leads to formation of a BCR-ABL transcript that is translated to form a 210 kd protein present in virtually all cases of CML (4-6). In 90% of the cases, the fusion gene results from a reciprocal translocation involving chromosomes 9 and 22 producing a cytogenetically distinct small acrocentric chromosome called the Philadelphia (Ph¹) chromosome (7-12), Fig. 8. However, standard cytogenetics does not have the resolution to distinguish closely spaced breakpoints, such as those characteristic of CML and acute lymphocytic leukemia (ALL), and misses fusions produced by more complex rearrangements. Mapping and cloning of the breakpoint regions in both genes has lead to molecular techniques capable of demonstrating BCR-ABL fusion in CML cases where the Ph¹ chromosome could not be detected cytogenetically (13-16). Southern analysis for BCR rearrangements has become the standard for diagnosis of CML. More recently, fusion has been detected by in vitro amplification of a cDNA transcript copied from CML mRNA using reverse transcriptase (17-23). That technique permits detection of BCR-ABL 25 transcript from CML cells present at low frequencies. Both of those techniques utilize nucleic acid obtained from cell populations so that correlation between genotype and phenotype for individual cells is not possible.

30 Described herein are chromosome-specific reagents and methods to detect genetic rearrangements, such as those exemplified herein for the BCR-ABL fusion, that supply information unavailable by existing techniques.

35 SUMMARY OF THE INVENTION

This invention concerns methods of staining chromosomal material based upon nucleic acid sequence that employ one or more nucleic acid probes, as set forth in claim 1. Said methods produce staining patterns that can be tailored 40 for specific cytogenetic analyses. The methods use nucleic acid probes that stain chromosomal material with reliable signals. Such probes are appropriate for in situ hybridization. Preferred nucleic acid probes for certain applications of this invention are those of sufficient complexity to stain reliably each of two or more target sites.

The invention provides methods for staining chromosomal material. The probe compositions of this invention at the current state of hybridization techniques are typically of high complexity, usually greater than about 50 kb of complexity, the complexity depending upon the application for which the probe is designed. In particular, chromosome 45 specific staining reagents are provided which comprise heterogeneous mixtures of nucleic acid fragments, each fragment having a substantial fraction of its sequences substantially complementary to a portion of the nucleic acid for which specific staining is desired — the target nucleic acid, preferably the target chromosomal material. In general, the nucleic acid fragments are labeled by means as exemplified herein and indicated infra. However, the nucleic acid fragments need not be directly labeled in order for the binding of probe fragments to the target to be detected; for example, such nucleic acid binding can be detected by anti-RNA/DNA duplex antibodies and antibodies to thymidine 50 dimers. The nucleic acid fragments of the heterogenous mixtures include double-stranded or single-stranded RNA or DNA.

This invention concerns chromosome specific reagents and methods of staining targeted chromosomal material that is in the vicinity of a suspected genetic rearrangement.

55 As used herein the term "genetic rearrangement" refers to a translocation.

When such a genetic rearrangement is associated with a disease, such chromosome specific reagents are referred to as disease specific reagents or probes. When such a genetic rearrangement is associated with cancer, such reagents are referred to as tumor specific reagents or probes.

This invention uses nucleic acid probes that reliably stain targeted chromosomal materials in the vicinity of one or more suspected genetic rearrangements. Such nucleic acid probes useful for the detection of genetic rearrangements are typically of high complexity. Such nucleic acid probes preferably comprise nucleic acid sequences that are substantially homologous to nucleic acid sequences in chromosomal regions that flank and/or extend partially or fully across breakpoints associated with genetic rearrangements.

5 This invention further provides for methods to distinguish between cytogenetically similar but genetically different chromosomal rearrangements.

Specifically herein exemplified are chromosome specific reagents and methods to detect genetic translocations, that produce the BCR-ABL fusion which is diagnostic for chronic myelogenous leukemia (CML). Such chromosome 10 specific reagents for the diagnosis of CML contain nucleic acid sequences which are substantially homologous to chromosomal sequences in the vicinity of the translocation breakpoint regions of chromosomal regions 9q34 and 22q11 associated with CML.

Those reagents produce a staining pattern which is distinctively altered when the BCR-ABL fusion characteristic 15 of CML occurs. Figure 11 graphically demonstrates a variety of staining patterns which, along with other potential staining patterns, are altered in the presence of a genetic rearrangement, such as, the BCR-ABL fusion.

The presence of a genetic rearrangement can be determined by applying the reagents of this invention according to methods herein described and observing the proximity of and/or other characteristics of the signals of the staining 20 patterns produced.

Preferably, the chromosome specific reagents used to detect CML of this invention comprise nucleic acid sequences having a complexity of from about 50 kilobases (kb) to about 1 megabase (Mb), more preferably from about 50 kb to about 750 kb, and still more preferably from about 200 kb to about 400 kb.

This invention further provides for methods of distinguishing between suspected genetic rearrangements that occur 25 in relatively close proximity in a genome wherein the chromosome specific reagents comprise nucleic acid sequences substantially homologous to nucleic acid sequences in the vicinity of said suspected genetic rearrangements. An example of such a differentiation between two potential genetic rearrangements is the differential diagnosis of CML from acute lymphocytic leukemia (ALL).

This invention still further provides methods for producing staining patterns in a patient who is afflicted with a disease associated genetic rearrangement, such as those associated with the BCR-ABL fusion in CML, wherein said staining patterns are predictive and/or indicative of the response of a patient to various therapeutic regimens, such as 30 chemotherapy, radiation, surgery, and transplantation, such as bone marrow transplantation. Such staining patterns can be useful in monitoring the status of such a patient, preferably on a cell by cell basis, and can be predictive of a disease recurrence for a patient that is in remission. Computer assisted microscopic analysis can assist in the interpretation of staining patterns of this invention, and the invention provides for methods wherein computer assisted microscopic analysis is used in testing patient cells on a cell by cell basis, for e.g., to search for residual disease in a 35 patient.

Still further, this invention provides for methods to determine the molecular basis of genetic disease, and to detect specific genetically based diseases.

Still further, this invention provides for methods for detecting contiguous gene syndromes comprising the in situ 40 hybridization of nucleic acid probes which comprise sequences which are substantially homologous to nucleic acid sequences characteristic of one or more components of a contiguous gene syndrome. Representative of such a contiguous gene syndrome is Down syndrome.

Also provided are methods of simultaneously detecting genetic rearrangements of multiple loci in a genome comprising in situ hybridization of high complexity nucleic acid probes comprising nucleic acid sequences that are substantially homologous to nucleic acid sequences in multiple loci in a genome.

45 Still further provided are methods of searching for genetic rearrangements in a genome. For example, conventional banding analysis may indicate an abnormality in a chromosomal region of a genome under examination. Methods of this invention may include the application of nucleic acid probes, produced from the vicinity of that chromosomal region of a normal genome, by in situ hybridization to cells containing the abnormality to detail the exact location and kind of genetic rearrangement of said abnormality by observation of the staining patterns so produced.

50 The invention still further provides for high complexity nucleic acid probes which have been optimized for rapid, efficient and automated detection of genetic rearrangements.

One way to produce a probe of high-complexity is to pool several or many clones, for example, phage, plasmid, 55 cosmid, and /or YAC clones, among others, wherein each clone contains an insert that is capable of hybridizing to some part of the target in a genome. Another way to produce such a probe is to use the polymerase chain reaction (PCR).

Heterogeneous in reference to the mixture of labeled nucleic acid fragments means that the staining reagents comprise many copies each of fragments having different sequences and/or sizes (e.g., from the different DNA clones pooled to make the probe). In preparation for use, these fragments may be cut, randomly or specifically, to adjust the

size distribution of the pieces of nucleic acid participating in the hybridization reaction.

As discussed more fully below, preferably the heterogeneous probe mixtures are substantially free from nucleic acid sequences with hybridization capacity to non-target nucleic acid. Most of such sequences bind to repetitive sequences which are shared by the target and non-target nucleic acids, that is, shared repetitive sequences.

Methods to remove undesirable nucleic acid sequences and/or to disable the hybridization capacity of such sequences are discussed more fully below. [See Section II]. Such methods include but are not limited to the selective removal or screening of shared repetitive sequences from the probe; careful selection of nucleic acid sequences for inclusion in the probe; blocking shared repetitive sequences by the addition of unlabeled genomic DNA, or, more carefully selecting nucleic acid sequences for inclusion in the blocking mixture; incubating the probe mixture for sufficient time for reassociation of high copy repetitive sequences, or the like.

The staining reagents of the invention are applied to interphase chromosomal DNA by *in situ* hybridization, and the chromosomes are identified or classified, i.e., karyotyped, by detecting the presence of the label, on the nucleic acid fragments comprising the staining reagent.

The invention includes chromosome staining reagents for the total genomic complement of chromosomes, staining reagents specific to single chromosomes, staining reagents specific to subsets of chromosomes, and staining reagents specific to subregions within single or multiple chromosomes. The term "chromosome-specific," is understood to encompass all of these embodiments of the staining reagents of the invention. The term is also understood to encompass staining reagents made from and directed against both normal and abnormal chromosome types.

A preferred method of making the chromosome-specific staining reagents of the invention includes: 1) isolating chromosomal DNA from a particular chromosome type or target region or regions in the genome, 2) amplifying the isolated DNA to form a heterogeneous mixture of nucleic acid fragments, 3) disabling the hybridization capacity of or removing shared repeated sequences in the nucleic acid fragments, and 4) labeling the nucleic acid fragments to form a heterogeneous mixture of labeled nucleic acid fragments. As described more fully below, the ordering of the steps for particular embodiments varies according to the particular means adopted for carrying out the steps.

The present invention addresses problems associated with karyotyping chromosomes, especially for diagnostic and dosimetric applications. In particular, the invention overcomes problems which arise because of the lack of stains that are sufficiently chromosome-specific by providing reagents comprising heterogeneous mixtures of nucleic acid fragments that can be hybridized to the target DNA and/or RNA, e.g., the target chromosomes, target subsets of chromosomes, or target regions of specific chromosomes. The staining technique of the invention opens up the possibility of rapid and highly sensitive detection of genetic rearrangements, in interphase cells using standard clinical and laboratory equipment and improved analysis using automated techniques. It has direct application in genetic screening, cancer diagnosis, and biological dosimetry.

This invention further specifically provides for methods for staining fetal chromosomal material.

Still further, the invention provides for a non-embryo-invasive method of karyotyping the chromosomal material of fetal cells, wherein the fetal cells have been separated from maternal blood. Such fetal cells are preferably leukocytes and/or cytotrophoblasts. Exemplary nucleic acid probes are high complexity probes chromosome-specific for chromosome types 13, 18 and/or 21. Representative probes comprise chromosome-specific Bluescribe (Registered Trade Mark) plasmid libraries from which a sufficient number of shared repetitive sequences have been removed or the hybridization capacity thereof has been disabled prior to and/or during hybridization with the target fetal chromosomes.

This application discloses test kits comprising appropriate nucleic acid probes for use in tumor cytogenetics, in the detection of disease related loci, in the analysis of translocations, and for biological dosimetry.

This application further discloses prenatal screening kits comprising appropriate nucleic acid probes, including test kits comprising high complexity probes for the detection of genetic rearrangements, and specifically for those producing the BCR-ABL fusion characteristic of CML.

The methods of this invention permit staining of chromosomal material with patterns appropriate for a desired application. The pattern may extend over some regions of one or more chromosomes, or over some or all the chromosomes of a genome and multiple sections, distinguishable by multiple colors.

Alternatively, the pattern may be focused on a particular portion or portions of a genome, such as a portion or portions potentially containing a breakpoint that is diagnostically or prognostically important for one or more tumors, or on those portions of chromosomes having significance for prenatal diagnosis.

The staining patterns may be adjusted for the analysis method employed, for example, either a human observer or automated equipment, such as, flow cytometers or computer-assisted microscopy. The patterns may be chosen to be appropriate for analysis of condensed chromosomes or dispersed chromosomal material.

The invention further provides for automated means of detecting and analyzing chromosomal abnormalities, particularly genetic rearrangements, as indicated by the staining patterns produced according to this invention.

Another object of the present invention is to provide an alternative method to currently available techniques for preparing and applying non-selfcomplementary single stranded DNA hybridization probes.

Another object of the invention is to improve the signal-to-noise ratios attainable in *in situ* hybridization by reducing

nonspecific and mismatched binding of probe.

Another object of the invention is to provide a method of denaturing double stranded target DNA for application of hybridization probe which minimizes single stranded regions available for hybridization that are noncomplementary to probe sequences.

5 DNA fragments from which probes can be constructed by treating with a restriction endonuclease which generates a collection of restriction fragments having "sticky" ends, or staggered cuts, characteristic of the endonuclease used. That is, the two fragment ends introduced by a cut each consist of a protruding strand and a recessed strand. The restriction fragments are inserted into vectors which have been engineered to accept that type of restriction fragment; and the vectors are transfected into host organisms which are grown to increase the number of restriction fragments.
 10 Next the vectors are separated from the host organisms, and the restriction fragments are excised and separated from the vectors. On each end of the restriction fragments the recessed strands are digested by an appropriate exonuclease. Digestion is not allowed to go to completion. The exonuclease treated restriction fragments are then used as template/primers for DNA polymerase which replaces the digested strand in the presence of a labeled precursor. Examples of enzymes suitable for this process are exonuclease III followed by treatment with the large fragment of DNA polymerase
 15 I; or T4 DNA polymerase, which can perform both functions by changes in reaction conditions. After synthesis is completed, the restriction fragments are broken into smaller fragments such that the labeled portions of the original restriction fragment remain substantially intact. The smaller fragments are denatured, and the labeled strands are separated from the unlabeled strands to form the hybridization probes.

Under this method of using single-stranded probes, before application of the hybridization probe to the target DNA,
 20 the target DNA is first treated with the same restriction endonuclease used to excise the probe DNA from the cloning vector. This treatment breaks the target DNA into a collection of restriction fragments having tails at each end characteristic of the restriction endonuclease. Next the target DNA is treated with an exonuclease which removes the recessed strand, thereby exposing single stranded DNA in the vicinity of the cut introduced by the restriction endonuclease. Finally, the hybridization probe is applied to the target DNA, e.g., using standard *in situ* hybridization protocols, as
 25 described more fully below.

An important feature of the single stranded probe method is treating the cloned probe DNA and the target DNA with the same restriction endonuclease. This ensures that the single stranded DNA of the target is complementary to the labeled strand of the probe. Of course many segments of the target in addition to the correct binding sites will be made single stranded because there are many restriction cuts, but there will be much less total single stranded target
 30 than would be made by indiscriminate denaturation. In addition, target DNA rendered single stranded in this manner cannot reanneal with itself and thus block access to the probe.

Another important (but not critical) feature of such a method is the selection of a label which permits labeled strands to be separated from unlabeled strands. Preferably precursors are labeled by biotinylation, and the labeled strands are separated from unlabeled strands by affinity chromatography.
 35

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, B and C and Figures 2A and 2B illustrate the hybridization of a chromosome-specific 21 library to human metaphase spread wherein the inserts were cloned in Lambda phage Charon 21A. The hybridization capacity of the high copy repetitive sequences in the library was reduced by the addition of unlabeled genomic DNA to the hybridization mixture. The probe was labeled with biotin, which was detected with green FITC-avidin (fluorescein isothiocyanate avidin). All of the DNA in the chromosomes was stained with the blue fluorescent dye DAPI (4,6-diamidino-2-phenylindole).

Figure 1A is a binary image of the DAPI stain in the human metaphase spread obtained by using a TV camera attached to a fluorescence microscope. Filters appropriate for DAPI visualization were used. Computer processing of the image shows all portions above a chosen threshold intensity as white, and the rest as black.

Figure 1B is a binary image of the FITC staining of the same human metaphase spread as in Figure 1A. The image was processed as in Figure 1A but the filter was changed in the microscope such that the FITC attached to the probe is visible rather than the DAPI.

Figure 1C is a binary image of the chromosome 21s alone, nonspecifically stained objects (which are smaller) having been removed by standard image processing techniques on the binary image of Figure 1B.

Figure 2A is a color photograph of the DAPI stain in a human metaphase spread which was prepared and hybridized contemporaneously with the spread shown in the computer generated binary images of Figures 1A, B and C.

Figure 2B is a color photograph of the fluorescein attached to the DNA probe in the same human metaphase spread as shown in Figure 2A. It was obtained by changing the filters in the fluorescence microscope to excite fluorescein rather than DAPI. The photograph is comparable to the binary image of Figure 1B.

Figure 3 is a photograph of a human metaphase spread prepared and hybridized contemporaneously with the spreads shown in Figures 1A, B and C and 2A and B. The procedures used were the same except that PI (propidium

iodide) instead of DAPI, was used to stain all the chromosomes. Both PI and fluorescein stains can be viewed with the same microscope filters. Color film was used such that the propidium iodide counterstain appears red and the fluorescein of the probe appears yellow on the color film.

Figure 4A shows the hybridization of the chromosome 4-specific library in Bluescribe plasmids (the library pBS-4) to a human metaphase spread wherein no unlabeled human genomic DNA was used, and wherein the hybridization mixture was applied immediately after denaturation. Both copies of chromosome 4 are seen as slightly brighter than the other chromosomes. The small arrows indicate regions that are unstained with the probe. As in Figure 3 and as in the rest of the Figures below, PI is the counterstain and fluorescein is used to label the probe.

Figure 4B shows the hybridization of pBS-4 to a human metaphase spread wherein unlabeled human genomic DNA was used during the hybridization ($Q = 2$ of genomic DNA; the meaning of Q is explained *infra*). Quantitative image analysis shows that the intensity per unit length of the chromosome 4s is about 20X that of the other chromosomes. The chromosome 4s are yellow; the other chromosomes are red due to the propidium iodide counterstain. Two layers of avidin-fluorescein isothiocyanate have been used to make the target chromosomes sufficiently bright to be measured accurately. However, the number 4 chromosomes can be recognized easily after a single layer is applied.

Figure 4C shows the same spread as in Figure 4B but through a filter that passes only the fluorescein isothiocyanate fluorescence.

Figure 4D shows the detection of a radiation-induced translocation (arrows) involving chromosome 4s in a human metaphase spread wherein pBS-4 specific libraries are used. The contrast ratio is about 5X.

Figure 4E shows that normal and two derivative chromosomes resulting from a translocation between chromosome 4 and 11 (in cell line RS4; 11) can be detected by the compositions and methods of this invention in interphase nuclei. They appear as three distinct domains.

Figure 4F shows the hybridization of the chromosome 21-specific library in Bluescribe plasmids (the library pBS-21) to a metaphase spread of a trisomy 21 cell line. A small amount of hybridization is visible near the centromeres of the other acrocentric chromosomes.

Figure 4G shows the same hybridization as in Figure 4F but with interphase nuclei. Clearly shown are the three chromosome 21 domains.

Figure 4H shows the hybridization with a pool of 120 single copy probes from chromosome 4 to a human metaphase spread. The number 4 chromosomes are indicated by arrows.

Figure 5 shows the hybridization of a yeast artificial chromosome (YAC) clone containing a 580 kb insert of human DNA to a human metaphase spread. A yellow fluorescein band on each of the chromosome 12s (at 12q21.1) is visible against the propidium iodide counterstain.

Figure 6 shows the hybridization of DNA from a human/hamster hybrid cell containing one copy of human chromosome 19 to a human metaphase spread. A little to the right of the photograph's center are the two chromosome 19s which are brighter than the other chromosomes in the spread.

Figure 7 illustrates a representative method of using the polymerase chain reaction (PCR) to produce probes of this invention which are reduced in repetitive sequences.

Figure 8 illustrates the locations of probes to the CML breakpoint and corresponding pattern of staining in both normal and CML metaphase and interphase nuclei.

The left side shows schematic representations of the BCR gene on chromosome 22, the ABL gene of chromosome 9, and the BCR-ABL fusion gene on the Philadelphia chromosome. Also shown are the locations of CML breakpoints and their relation to the probes (32). The right shows hybridization patterns expected for the c-hu-ABL and PEM12 probes to normal and CML metaphase spreads and interphase nuclei.

Figure 9 shows fluorescence in-situ hybridization (FISH) in metaphase spreads and interphase nuclei. Panels A and B show ABL and BCR hybridization to normal metaphase spreads. The ABL signal (A) is localized to the telomeric portion of 9q and the BCR signal (B) is localized near the centromere of 22q. Panel C shows that abl staining is localized to the telomeric region of Philadelphia chromosome in a case of CML with 46XY, t(9; 22)(q34; q11). Panel D shows that abl staining is interstitial on the derivative 22 chromosome arising from an insertional event in a case of CML with 46XY ins (22; 9)(q11; q34). Panel E illustrates that the K562 cell line presents multiple signals localized to a region of the interphase nucleus. Identical staining pattern was seen with BCR probe indicating BCR -ABL fusion gene amplification. Panel F presents a metaphase spread from the K562 cell line showing fusion gene amplification localized to a single chromosome.

Figure 10 illustrates fluorescence in-situ hybridization in CML interphase nuclei with ABL-(red)-and-BCR-(green) probes visualized simultaneously through a double band pass filter. Cells from a CML patient show the red-green (yellow) signals resulting from the hybridization to the BCR-ABL fusion gene and single red and green hybridization signals to the normal BCR and ABL genes on chromosomes 22 and 9.

Figure 11 illustrates some exemplary probe strategies for detection of structural aberrations. The design of the binding pattern, colors etc., of the probe can be optimized for detection of genetic abnormalities in metaphase and/or interphase cells. Different patterns may have advantages for particular applications. The drawings in Figure 11 illustrate

some of the patterns useful for detection of some abnormalities. The examples are representative and not meant to be exhaustive; different patterns can be combined to allow for the detection of multiple abnormalities in the same cell.

In the drawings of Figure 11, the metaphase chromosomes are shown with probe bound to both chromatids. The interphase nuclei are pictured to be in a stage of the cell cycle prior to replication of the portion of the chromosome to which the probe binds; thus there is only one chromatid for each interphase chromosome. When the probe binding is restricted to only a portion of a chromosome, the signal is indicated as either a black or white circle. Such a representation is employed to indicate different colors or otherwise distinguishable characteristics of the staining. Patterns containing more than two distinguishable characteristics (three colors, different ratios of colors etc.) permit more complex staining patterns than those illustrated. Chromosomal locations of the breakpoints in the DNA are indicated with horizontal lines next to the abnormal chromosomes.

- a. Section a) represents the use of a probe which stains a whole chromosome. Such a probe can be used to detect a translocation that occurs anywhere along the chromosome. The color photograph of Figure 12 shows use of such a stain for chromosome 22 to detect a translocation, in this case that which occurs with CML. Such an approach to staining is not very useful in interphase nuclei since the region of the nucleus that is stained is relatively large; overlaps in the stained regions can make interpretation difficult in many nuclei.
- b. Section b) represents the reduction of the stained region of the chromosome shown in a) to that in the vicinity of a breakpoint, providing information focused on events in that region. The staining pattern can be continuous or discontinuous across the breakpoint, just so that some binding is on both sides of the breakpoint. Such a staining pattern requires only one "color", but gives no information about which other genomic region may be involved in the exchange.
- c. Section c) represents the use of a probe which binds to sequences which come together as a result of the rearrangement and allows for the detection in metaphase and interphase cells. In this case the different sequences are stained with different "colors". Such a staining pattern is that used in the examples of Section VIII of this application.
- d. Section d) represents an extension of c) by including staining of both sides of both breakpoints involved in the rearrangement. Different "colors" are used as indicated. The additional information supplied by the more complex staining pattern may assist with interpretation of the nuclei. It might also permit recognition of an apparent insertion event as discussed herein.
- e. Section e) represents the detection of an inversion in one homologue of a chromosome.
- f. Section f) represents a staining pattern useful in the detection of a deletion. A deletion could also be detected with a probe that stains only the deleted region; however, lack of probe binding may be due to reasons other than deletion of the target sequence. The flanking regions stained a different "color" serve as controls for hybridization.

Figure 12 illustrates a staining pattern to detect a rearrangement by staining a whole chromosome, in this case a rearrangement of chromosome 22 associated with CML. The metaphase spread of this figure is from a CML cell that has been stained with a probe which binds all along chromosome 22. Probe-stained regions appear yellow. The rest of the DNA has been stained with the red-fluorescing chemical stain propidium iodide. The entirely yellow chromosome is a normal copy of chromosome 22. Just below said normal chromosome 22 is the Philadelphia chromosome, a small part yellow and part red chromosome. Below and to the right of the Philadelphia chromosome is the abnormal chromosome 9 (red) with the distal part of chromosome 22 (yellow) attached. The photograph of this figure illustrates the staining pattern represented in part a) of the previous figure.

DETAILED DESCRIPTION OF THE INVENTION

This invention concerns the use of nucleic acid probes to stain targeted chromosomal material in patterns which can extend along one or more whole chromosomes, and/or along one or more regions on one or more chromosomes, including patterns which extend over an entire genome. The staining reagents of this invention facilitate the microscopic and/or flow cytometric identification of normal and aberrant chromosomes and provide for the characterization of the genetic nature of particular abnormalities, such as, genetic rearrangements. The term "chromosome-specific" is herein defined to encompass the terms "target specific" and "region specific", that is, when the staining composition is directed to one chromosome, it is chromosome-specific, but it is also chromosome-specific when it is directed, for example, to multiple regions on multiple chromosomes, or to a region of only one chromosome, or to regions across the entire genome. The term chromosome-specific originated from the use of recombinant DNA libraries made by cloning DNA from a single normal chromosome type as the source material for the initial probes of this invention. Libraries made from DNA from regions of one or more chromosomes are sources of DNA for probes for that region or those regions of the genome. The probes produced from such source material are region-specific probes but are also encompassed within the broader phrase "chromosome-specific" probes. The term "target specific" is interchangeably used herein

with the term "chromosome-specific".

The word "specific" as commonly used in the art has two somewhat different meanings. The practice is followed herein. "Specific" may refer to the origin of a nucleic acid sequence or to the pattern with which it will hybridize to a genome as part of a staining reagent. For example, isolation and cloning of DNA from a specified chromosome results in a "chromosome-specific library". [Eg., Van Dilla et al., "Human Chromosome-Specific DNA Libraries: Construction and Availability," Biotechnology, 4: 537 (1986).] However, such a library contains sequences that are shared with other chromosomes. Such shared sequences are not chromosome-specific to the chromosome from which they were derived in their hybridization properties since they will bind to more than the chromosome of origin. A sequence is "chromosome-specific" if it binds only to the desired portion of a genome. Such sequences include single-copy sequences contained in the target or repetitive sequences, in which the copies are contained predominantly in the target.

"Chromosome-specific" in modifying "staining reagent" refers to the overall hybridization pattern of the nucleic acid sequences that comprise the reagent. A staining reagent is chromosome-specific if useful contrast between the target and non-target chromosomal material is achieved (that is, that the target can be adequately visualized).

A probe is herein defined to be a collection of nucleic acid fragments whose hybridization to the target can be detected. The probe is labeled as described below so that its binding to the target can be visualized. The probe is produced from some source of nucleic acid sequences, for example, a collection of clones or a collection of polymerase chain reaction (PCR) products. The source nucleic acid may then be processed in some way, for example, by removal of repetitive sequences or blocking them with unlabeled nucleic acid with complementary sequence, so that hybridization with the resulting probe produces staining of sufficient contrast on the target. Thus, the word probe may be used herein to refer not only to the detectable nucleic acid, but also to the detectable nucleic acid in the form in which it is applied to the target, for example, with the blocking nucleic acid, etc. The blocking nucleic acid may also be mentioned separately. What "probe" refers to specifically should be clear from the context in which the word is used.

When two or more nucleic acid probes of this invention are mixed together, they produce a new probe which when hybridized to a target according to the methods of this invention, produces a staining pattern that is a combination of the staining patterns individually produced by the component probes thereof. Thus, the terms "probe" and "probes" (that is, the singular and plural forms) can be used interchangeably within the context of a staining pattern produced. For example, if one probe of this invention produces a dot on chromosome 9, and another probe produces a band on chromosome 11, together the two probes form a probe which produces a dot/band staining pattern.

The term "labeled" is herein used to indicate that there is some method to visualize the bound probe, whether or not the probe directly carries some modified constituent. Section III infra describes various means of directly labeling the probe and other labeling means by which the bound probe can be detected.

The terms "staining" or "painting" are herein defined to mean hybridizing a probe of this invention to a genome or segment thereof, such that the probe reliably binds to the targeted chromosomal material therein and the bound probe is capable of being visualized. The terms "staining" or "painting" are used interchangeably. The patterns resulting from "staining" or "painting" are useful for cytogenetic analysis, more particularly, molecular cytogenetic analysis. The staining patterns facilitate the microscopic and/or flow cytometric identification of normal and abnormal chromosomes and the characterization of the genetic nature of particular abnormalities. Section III infra describes methods of rendering the probe visible. Since multiple compatible methods of probe visualization are available, the binding patterns of different components of the probe can be distinguished-for example, by color. Thus, this invention is capable of producing any desired staining pattern on the chromosomes visualized with one or more colors (a multicolor staining pattern) and/or other indicator methods. The term "staining" as defined herein does not include the concept of staining chromosomes with chemicals as in conventional karyotyping methods although such conventional stains may be used in conjunction with the probes of this invention to allow visualization of those parts of the genome where the probe does not bind. The use of DAPI and propidium iodide for such a purpose is illustrated in the figures.

The phrase "high complexity" is defined herein to mean that the probe, thereby modified contains on the order of 50,000 (50 kb) or greater, up to many millions or several billions, of bases of nucleic acid sequences which are not repeated in the probe. For example, representative high complexity nucleic acid probes of this invention can have a complexity greater than 50 kb, greater than 100,000 bases (100 kb), greater than 200,000 (200 kb), greater than 500,000 bases (500 kb), greater than one million bases (1 Mb), greater than 2 Mb, greater than 10 Mb, greater than 100 Mb, greater than 500 Mb, greater than 1 billion bases and still further greater than several billion bases.

The term "complexity" is defined herein according to the standard for nucleic acid complexity as established by Britten et al., Methods of Enzymol., 29: 363 (1974). See also Cantor and Schimmel, Biophysical Chemistry: Part III: The Behavior of Biological Macromolecules, at 1228-1230 (Freeman and Co. 1980) for further explanation and exemplification of nucleic acid complexity.

The complexity preferred for a probe composition of this invention is dependent upon the application for which it is designed. In general, the larger the target area, the more complex is the probe. It is anticipated that the complexity of a probe needed to produce a desired pattern of landmarks on a chromosome will decrease as hybridization sensitivity increases, as progress is made in hybridization technology. As the sensitivity increases, the reliability of the signal from

smaller target sites will increase. Therefore, whereas from about a 40 kb to about a 100 kb target sequence may be presently necessary to provide a reliable, easily detectable signal, smaller target sequences should provide reliable signals in the future. Therefore, as hybridization sensitivity increases, a probe of a certain complexity, for example, 100 kb, should enable the user to detect considerably more loci in a genome than are presently reliably detected; thus, 5 more information will be obtained with a probe of the same complexity. The term "complexity" therefore refers to the complexity of the total probe no matter how many visually distinct loci are to be detected, that is, regardless of the distribution of the target sites over the genome.

As indicated above, with current hybridization techniques it is possible to obtain a reliable, easily detectable signal with a probe of about 40 kb to about 100 kb (eg. the probe insert capacity of one or a few cosmids) targeted to a compact point in the genome. Thus, for example, a complexity in the range of approximately 100 kb now permits hybridization to both sides of a tumor-specific translocation. The portion of the probe targeted to one side of the breakpoint can be labeled differently from that targeted to the other side of the breakpoint so that the two sides can be differentiated with different colors, for example. Proportionately increasing the complexity of the probe permits analysis of multiple compact regions of the genome simultaneously. The conventional banding patterns produced by chemical stains may be replaced according to this invention with a series of probe-based, color coded (for example), reference points along each chromosome or significant regions thereof.

Uniform staining of an extended contiguous region of a genome, for example, a whole chromosome, requires a probe complexity proportional to but substantially less than, the complexity of the target region. The complexity required is only that necessary to provide a reliable, substantially uniform signal on the target. Section V.B, infra, demonstrates 20 that fluorescent staining of human chromosome 21, which contains about 50 megabases (Mb) of DNA, is sufficient with a probe complexity of about 1 Mb. Figure 4H illustrates hybridization of about 400 kb of probe to human chromosome 4, which contains about 200 Mb of DNA. In that case, gaps between the hybridization of individual elements of the probe are visible. Figures 4B and 4F demonstrate the results achieved with probes made up of entire libraries for 25 chromosomes 4 and 21, respectively. The chromosomes are stained much more densely as shown in Figures 4B and 4F than with the lower complexity probe comprising single-copy nucleic acid sequences used to produce the pattern of Figure 4H.

Increasing the complexity beyond the minimum required for adequate staining is not detrimental as long as the total nucleic acid concentration in the probe remains below the point where hybridization is impaired. The decrease in concentration of a portion of a sequence in the probe is compensated for by the increase in the number of target sites. 30 In fact, when using double-stranded probes, it is preferred to maintain a relatively low concentration of each portion of sequence to inhibit reassociation before said portion of sequence can find a binding site in the target.

The staining patterns of this invention comprise one or more "bands". The term "band" is herein defined as a reference point in a genome which comprises a target nucleic acid sequence bound to a probe component, which duplex is detectable by some indicator means, and which at its narrowest dimension provides for a reliable signal under 35 the conditions and protocols of the hybridization and the instrumentation, among other variables, used. A band can extend from the narrow dimension of a sequence providing a reliable signal to a whole chromosome to multiple regions on a number of chromosomes.

The probe-produced bands of this invention are to be distinguished from bands produced by chemical staining as indicated above in the Background. The probe-produced bands of this invention are based upon nucleic acid sequence 40 whereas the bands produced by chemical staining depend on natural characteristics of the chromosomes, but not the actual nucleic acid sequence. Further, the banding patterns produced by chemical staining are only interpretable in terms of metaphase chromosomes whereas the probe-produced bands of this invention are useful both for metaphase and interphase chromosomes.

One method of forming the probes of the present invention is to pool many different low complexity probes. Such 45 a probe would then comprise a "heterogeneous mixture" of individual cloned sequences. The number of clones required depends on the extent of the target area and the capacity of the cloning vector. If the target is made up of several discrete, compact loci, that is, single spots at the limit of microscopic resolution, then about 40 kb, more preferably 100 kb, for each spot gives a reliable signal given current techniques. The portion of the probe for each spot may be made up from, for example, a single insert from a yeast artificial chromosome (YAC), from several cosmids each containing 50 35-40 kb or probe sequence, or from about 25 plasmids each with 4 kb of sequence.

Representative heterogeneous mixtures of clones exemplified herein include phage (Figures 1, 2 and 3), and plasmids-(Figure 4). Yeast-artificial-chromosomes-(YACS)-(Figure 5), and a single-human chromosome-in-an inter-species hybrid cell (Figure 6) are examples of high complexity probes for single loci and an entire chromosome that can be propagated as a single clone.

55 A base sequence at any point in the genome can be classified as either "single-copy" or "repetitive". For practical purposes the sequence needs to be long enough so that a complementary probe sequence can form a stable hybrid with the target sequence under the hybridization conditions being used. Such a length is typically in the range of several tens to hundreds of nucleotides.

A "single-copy sequence" is that wherein only one copy of the target nucleic acid sequence is present in the haploid genome. "Single-copy sequences" are also known in the art as "unique sequences". A "repetitive sequence" is that wherein there are more than one copy of the same target nucleic acid sequence in the genome. Each copy of a repetitive sequence need not be identical to all the others. The important feature is that the sequence be sufficiently similar to the other members of the family of repetitive sequences such that under the hybridization conditions being used, the same fragment of probe nucleic acid is capable of forming stable hybrids with each copy. A "shared repetitive sequence" is a sequence with some copies in the target region of the genome, and some elsewhere.

When the adjectives "single-copy", "repetitive", "shared repetitive", among other such modifiers, are used to describe sequences in the probe, they refer to the type of sequence in the target to which the probe sequence will bind. Thus, "a repetitive probe" is one that binds to a repetitive sequence in the target; and "a single-copy probe" binds to a single-copy target sequence.

Repetitive sequences occur in multiple copies in the haploid genome. The number of copies can range from two to hundreds of thousands, wherein the Alu family of repetitive DNA are exemplary of the latter numerous variety. The copies of a repeat may be clustered or interspersed throughout the genome. Repeats may be clustered in one or more locations in the genome, for example, repetitive sequences occurring near the centromeres of each chromosome, and variable number tandem repeats (VNTRs) [Nakamura et al, *Science*, 235: 1616 (1987)]; or the repeats may be distributed over a single chromosome [for example, repeats found only on the X chromosome as described by Bardoni et al., *Cytogenet. Cell Genet.*, 46: 575 (1987)]; or the repeats may be distributed over all the chromosomes, for example, the Alu family of repetitive sequences.

Herein, the terms repetitive sequences, repeated sequences and repeats are used interchangeably.

Shared repetitive sequences can be clustered or interspersed. Clustered repetitive sequences include tandem repeats which are so named because they are contiguous on the DNA molecule which forms the backbone of a chromosome. Clustered repeats are associated with well-defined regions of one or more chromosomes, e.g., the centromeric region. If one or more clustered repeats form a sizable fraction of a chromosome, and are shared with one or more non-target regions of the genome and are consequently removed from the heterogeneous mixture of fragments employed in the invention or the hybridization capacity thereof is disabled, perfect uniformity of staining of the target region may not be possible. That situation is comprehended by the use of the term "substantially uniform" in reference to the binding of the heterogeneous mixture of labeled nucleic acid fragments to the target.

Chromosome-specific staining of the current invention is accomplished by using nucleic acid fragments that hybridize to sequences specific to the target. These sequences may be either single-copy or repetitive, wherein the copies of the repeat occur predominantly in the target area. Figure 4H and the results of the work detailed in section V infra indicate that probes can be made of single-copy sequences. However, in probes such as that of Figure 4B, low-copy chromosome-specific repeats [Nakamura et al., and Bardoni et al., *supra*] may contribute to the hybridization as well.

If nucleic acid fragments complementary to non-target regions of the genome are included in the probe, for example, shared repetitive sequences or non-specific sequences, their hybridization capacity needs to be sufficiently disabled or their prevalence sufficiently reduced, so that adequate staining contrast can be obtained. Section V and Figure 4H show examples of hybridization with probes that contain pools of clones in which each clone has been individually selected so that it hybridizes to single-copy sequences or very low copy repetitive sequences. The remaining figures illustrate use of probes that contain fragments that could have hybridized to high-copy repetitive sequences, but which have had the hybridization capacity of such sequences disabled.

The nucleic acid probes of this invention need not be absolutely specific for the targeted portion of the genome. They are intended to produce "staining contrast". "Contrast" is quantified by the ratio of the stain intensity of the target region of the genome to that of the other portions of the genome. For example, a DNA library produced by cloning a particular chromosome, such as those listed in Table I, can be used as a probe capable of staining the entire chromosome. The library contains sequences found only on that chromosome, and sequences shared with other chromosomes. In a simplified (approximately true to life) model of the human genome, about half of the chromosomal DNA falls into each class. If hybridization with the whole library were capable of saturating all of the binding sites, the target chromosome would be twice as bright (contrast ratio of 2) as the others since it would contain signal from the specific and shared sequences in the probe, whereas the other chromosome would only have signal from the shared sequences. Thus, only a modest decrease in hybridization of the shared sequences in the probe would substantially enhance the contrast. Contaminating sequences which only hybridize to non-targeted sequences, for example, impurities in a library, can be tolerated in the probe to the extent that said sequences do not reduce the staining contrast below useful levels.

In reality all of the target sites may not be saturated during the hybridization, and many other mechanisms contribute to producing staining contrast, but this model illustrates one general consideration in using probes targeted at a large portion of a genome.

The required contrast depends on the application for which the probe is designed. When visualizing chromosomes and nuclei, etc., microscopically, a contrast ratio of two or greater is often sufficient for identifying whole chromosomes. In Figures 4D-F, the contrast ratio is 3-5. The smaller the individual segments of the target region, the greater the

contrast needs to be to permit reliable recognition of the target relative to the fluctuations in staining of the non-targeted regions. When quantifying the amount of target region present in a cell nucleus by fluorescence intensity measurements using flow cytometry or quantitative microscopy, the required contrast ratio is on the order of $1/T$ or greater on average for the genome, where T is the fraction of the genome contained in the targeted region. When the contrast ratio is equal to $1/T$, half of the total fluorescence intensity comes from the target region and half from the rest of the genome. For example, when using a high complexity probe for chromosome 1, which comprises about 10% of the genome, the required contrast ratio is on the order of 10, that is, for the chromosome 1 fluorescence intensity to equal that of the rest of the genome.

Background staining by the probe, that is, to the non-target region of the genome, may not be uniform. Figure 4F shows that a chromosome 21 specific probe contains probe fragments that hybridize weakly to compact regions near the centromeres of other acrocentric human chromosomes. This degree of non-specificity does not inhibit its use in the illustrated applications. For other applications, removal of or further disabling the hybridization capacity of the probe fragments that bind to these sequences may be necessary.

For other applications, repetitive sequences that bind to centromeres, for example, alpha-satellite sequences, and/or telomeres can be part of the chromosome-specific staining reagents wherein the target includes some or all of the centromeres and/or telomeres in a genome along with perhaps other chromosomal regions. Exemplary of such an application would be that wherein the staining reagent is designed to detect random structural aberrations caused by clastogenic agents that result in dicentric chromosomes and other structural abnormalities, such as translocations. Addition of sequences which bind to all centromeres in a genome, for example to the probe used to create the staining pattern of Figure 4D, would allow more reliable distinguishing between dicentrics and translocations.

Application of staining reagents of this invention to a genome results in a substantially uniform distribution of probe hybridized to the targeted regions of a genome. The distribution of bound probe is deemed "substantially uniform" if the targeted regions of the genome can be visualized with useful contrast. For example, a target is substantially uniformly stained in the case wherein it is a series of visually separated loci if most of the loci are visible in most of the cells.

"Substantial proportions" in reference to the base sequences of nucleic acid fragments that are complementary to chromosomal DNA means that the complementarity is extensive enough so that the fragments form stable hybrids with the chromosomal DNA under the hybridization conditions used. In particular, the term comprehends the situation where the nucleic acid fragments of the heterogeneous mixture possess some regions of sequence that are not perfectly complementary to target chromosomal material. The stringency can be adjusted to control the precision of the complementarity required for hybridization.

The phrase "metaphase chromosomes" is herein defined to mean not only chromosomes condensed in the metaphase stage of mitosis but includes any condensed chromosomes, for example, those condensed by premature chromosome condensation.

To disable the hybridization capacity of a nucleic acid sequence is herein sometimes abbreviated as "disabling the nucleic acid sequence".

The methods and reagents of this invention find a particularly appropriate application in the field of diagnostic cytogenetics, particularly in the field of diagnostic interphase cytogenetics. Detecting genetic rearrangements that are associated with a disease, such as cancer, are a specific application of the chromosome specific reagents and staining methods of this invention.

Contiguous gene syndromes are an example of the genetic rearrangements that the probes and methods of this invention can identify. Contiguous gene syndromes are characterized by the presence of several closely spaced genes which are in multiple and/or reduced copy number. Down syndrome is an example of a contiguous gene syndrome wherein an extra copy of a chromosomal region containing several genes is present.

Particularly described herein is the application of chromosome specific reagents and methods for detecting genetic rearrangements that produce the BCR-ABL fusion associated with CML. Such reagents are exemplary of disease specific, in this case tumor specific, probes which can be labeled, directly and/or indirectly, such that they are visualizable when bound to the targeted chromosomal material, which in the case of CML, is the vicinity of the translocation breakpoint regions of chromosomal regions 9q34 and 22q11 known to be associated with CML. In the examples provided in Section VIII of this application, the probes are labeled such that a dual color fluorescence is produced in the staining pattern of said probes upon in situ hybridization [fluorescent in situ hybridization (FISH)]; however, staining patterns can be produced in many colors as well as other types of signals, and any visualization means to signal the probe-bound-to its target can-be-used in the methods of this invention.

Section VIII herein describes representative methods and reagents of this invention to detect genetic rearrangements. The examples of Section VIII concern genetic rearrangements that produce the BCR-ABL fusion that is characteristic of CML. The approach in such examples is based on FISH with probes from chromosomes 9 and 22 that flank the fused BCR and ABL sequences in essentially all cases of CML (Figure 8). The probes when hybridized to the chromosomal material of both normal and abnormal cells produce staining patterns that are different as illustrated in Figures 8-12. The staining patterns produced by such exemplary probes are different in normal and abnormal cells;

the staining pattern present when the genetic rearrangement occurs is distinctively altered from that of the staining pattern shown by hybridizing the probes to chromosomal material that does not contain the genetic rearrangement. Further, staining patterns are distinctively different for one type of genetic rearrangement versus another. For example, the staining patterns produced upon hybridization of nucleic acid probes of this invention to chromosomal material containing a genetic rearrangement associated with ALL is distinctively different from that produced upon hybridization of such probes to chromosomal material containing the BCR-ABL fusion characteristic of CML. Thus, the methods and reagents of this invention provide for differential diagnosis of related diseases.

The examples of Section VIII provide for the diagnosis of CML based upon the proximity of the fluorescent signals in the staining patterns, and rely upon a 1 micron cutoff point for determination of the presence of a fusion. The proximity distance of signals is only one characteristic, among many others, of signals that can be used to detect the presence of a genetic rearrangement. Further, the proximity distance is dependent on the particular cell preparation techniques employed and the size of the nuclei therein, and for a particular cell preparation is relative depending on the distance between signals in normal and abnormal cells.

The staining patterns exemplified in the examples of Section VIII are representative of one type of probe strategy. Many other probe strategies can be employed. Figure 11 illustrates some other exemplary probe strategies for detecting genetic rearrangements, the patterns of which can be modified and optimized and otherwise varied to detect particular genetic rearrangements.

Use of other disease specific reagents of this invention would be analogous to the methods detailed in Section VIII for CML. For example, the diagnosis and study of acute lymphocytic leukemia (ALL) may be accomplished by replacing the BCR probe (PEM12) of Section VIII with a probe from the 5' end of the BCR gene. ALL is of particular interest because the Ph' chromosome is the most common cytogenetic abnormality in that disease, and the presence of such a chromosome is indicative of a very aggressive neoplasm.

The methods and reagents herein exemplified, particularly in Section VIII, provide for the means to distinguish between cytogenetically similar but genetically different diseases. "Cytogenetically" in that particular context refers to a similarity determined by conventional banding analysis. CML and ALL are in that context cytogenetically similar in that conventional banding analysis can not distinguish them because the breakpoints associated with each are so close together in the human genome.

Further, this invention provides methods and reagents that can be used in a cytogenetic research mode for the study of the molecular bases of genetic disease. For example, if an abnormality in a person's karyotype is noted by conventional banding analysis, the probes and reagents of this invention can be used to detect any genetic rearrangements in the vicinity of said abnormality. The underlying molecular basis of the abnormality can be determined by the methods and reagents of this invention, and the resulting differences at the genetic level may be indicative of different treatment plans and prognostically important. The underlying genetic rearrangements may be found to be consistently associated with a set of phenotypic characteristics in a population.

The following sections provide examples of making and using the staining compositions of this invention and are for purposes of illustration only and not meant to limit the invention in any way. Also included in the following description are examples of screening metaphase spreads. While such examples are not included within the scope of the present claims, they are considered to be useful in understanding and applying the present invention which relates to the detection of translocations in interphase cells. The following abbreviations are used.

| Abbreviations | |
|---------------|---|
| BN | bicarbonate buffer with NP-40 |
| DAPI | 4,6-diamidino-2-phenylindole |
| DCS | as in fluorescein-avidin DCS (a commercially available cell sorter grade of fluorescein Avidin D) |
| AAF | N-acetoxy-N-2-acetyl-aminofluorene |
| EDTA | ethylenediaminetetraacetate |
| FACS | fluorescence-activated cell sorting |
| FITC | fluorescein isothiocyanate |
| IB | isolation buffer |
| NP-40 | non-ionic detergent commercially available from Sigma as Nonidet P-40 (St. Louis, MO) |
| PBS | phosphate-buffered saline |
| PI | propidium iodide |
| PMSF | phenylmethylsulfonyl fluoride |
| PN buffer | mixture of 0.1 M NaH ₂ PO ₄ and 0.1 M Na ₂ HPO ₄ , pH 8; 0.1% NP-40 |
| PNM buffer | Pn buffer plus 5% nonfat dry milk (centrifuged); 0.02% Na azide |

(continued)

| Abbreviations | |
|---------------|-------------------------------------|
| SDS | sodium dodecyl sulfate |
| SSC | 0.15 M NaCl/0.015 M Na citrate, pH7 |
| VNTR | variable number tandem repeat |

I. Methods of Preparing Chromosome-Specific Staining ReagentsI.A. Isolation of Chromosome-Specific DNA and Formation of DNA Fragment Libraries.

The first step in a preferred method of making the compositions of the invention is isolating chromosome-specific DNA (which term includes target-specific and/or region-specific DNA, as indicated above, wherein specific refers to the origin of the DNA). This step includes first isolating a sufficient quantity of the particular chromosome type or chromosomal subregion to which the staining composition is directed, then extracting the DNA from the isolated chromosome(s) or chromosomal subregion(s). Here "sufficient quantity" means sufficient for carrying out subsequent steps of the method. Preferably, the extracted DNA is used to create a library of DNA inserts by cloning using standard genetic engineering techniques.

Preferred cloning vectors include, but are not limited to, yeast artificial chromosomes (YACS), plasmids, bacteriophages and cosmids. Preferred plasmids are Bluescribe plasmids; preferred bacteriophages are lambda insertion vectors, more preferably Charon 4A, Charon 21A, Charon 35, Charon 40 and GEM11; and preferred cosmids include Lawrist 4, Lawrist 5 and sCos1.

As indicated above, the DNA can be isolated from any source. Chromosome-specific staining reagents can be made from both plant and animal DNA according to the methods of this invention. Important sources of animal DNA are mammals, particularly primates or rodents wherein primate sources are more particularly human and monkey, and rodent sources are more particularly rats or mice, and more particularly mice.

1. Isolating DNA from an Entire Chromosome. A preferred means for isolating particular whole chromosomes (specific chromosome types) is by direct flow sorting [fluorescence-activated cell sorting (FACS)] of metaphase chromosomes with or without the use of interspecific hybrid cell systems. For some species, every chromosome can be isolated by currently available sorting techniques. Most, but not all, human chromosomes are currently isolatable by flow sorting from human cells, Carrano et al., "Measurement and Purification of Human Chromosomes by Flow Cytometry and Sorting," Proc. Natl. Acad. Sci., Vol. 76, pp. 1382-1384 (1979). Thus, for isolation of some human chromosomes, use of the human/rodent hybrid cell system may be necessary, see Kao, "Somatic Cell Genetics and Gene Mapping," InternationalReview of Cytology, Vol. 85, pp. 109-146 (1983), for a review, and Gusella et al., "Isolation and Localization of DNA Segments from Specific Human Chromosomes," Proc. Natl. Acad. Sci., Vol. 77, pp. 2829-2833 (1980). Chromosome sorting can be done by commercially available fluorescence-activated sorting machines, e.g., Becton Dickinson FACS-II, Coulter Epics V sorter, or special purpose sorters optimized for chromosome sorting or like instrument.

DNA is extracted from the isolated chromosomes by standard techniques, e.g., Marmur, "A Procedure for the Isolation of Deoxyribonucleic Acid from Micro-Organisms," J. Mol. Biol., Vol. 3, pp. 208-218 (1961); or Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, 1982) pp. 280-281. These references are incorporated by reference for their descriptions of DNA isolation techniques.

Generation of insert libraries from the isolated chromosome-specific DNA is carried out using standard genetic engineering techniques, e.g., Davies et al., "Cloning of a Representative Genomic Library of the Human X Chromosome After Sorting by Flow Cytometry," Nature, Vol. 293, pp. 374-376 (1981); Krumlauf et al., "Construction and Characterization of Genomic Libraries from Specific Human Chromosomes," Proc. Natl. Acad. Sci., Vol. 79, pp. 2971-2975 (1982); Lawn et al., "The Isolation and Characterization of Linked Delta-and-Beta-Globin Genes from a Cloned Library of Human DNA," Cell, Vol. 15, pp. 1157-1174 (1978); and Maniatis et al., "Molecular Cloning: A Laboratory Manual," (Cold Springs Harbor Laboratory, 1982), pp. 256-308; Van Dilla et al., id; Fuscoe, Gene, 52: 291 (1987); and Fuscoe et al., Cytogenet. Cell Genet., 43: 79 (1986).

Recombinant DNA libraries for each of the human chromosomes have been constructed by the National Laboratory Gene Library Project and are available from the American Type Culture Collection. [Van Dilla et al., Biotechnology, 4: 537 (1986).] Small insert-containing libraries were constructed by complete digestion of flow sorted human chromosome genomic DNA with HindIII or EcoRI and cloning into the Lambda insertion vector Charon 21A. The vector is capable of accepting human inserts of up to 9.1' kb in size. Thus, HindIII (or EcoRI) restriction fragments greater than 9.1' kb will not be recovered from these libraries. The observed average insert size in these libraries is approximately 4 kb. A representative list of the HindIII chromosome-specific libraries with their ATCC accession numbers are shown

in Table 1.

TABLE 1

| HUMAN CHROMOSOME - SPECIFIC GENOMIC LIBRARIES IN CHARON 21A VECTOR | | | |
|--|------------|--------|----------|
| | CHROMOSOME | ATCC # | LIBRARY |
| 5 | 1 | 57753 | LL01NS01 |
| 10 | 1 | 57754 | LL01NS02 |
| 15 | 2 | 57744 | LL02NS01 |
| 20 | 3 | 57751 | LL03NS01 |
| 25 | 4 | 57700 | LL04NS01 |
| 30 | 4 | 57745 | LL04NS02 |
| 35 | 5 | 57746 | LL05NS01 |
| 40 | 6 | 57701 | LL06NS01 |
| 45 | 7 | 57755 | LL07NS01 |
| 50 | 8 | 57702 | LL08NS02 |
| 55 | 9 | 57703 | LL09NS01 |
| 60 | 10 | 57736 | LL10NS01 |
| 65 | 11 | 57704 | LL11NS01 |
| 70 | 12 | 57756 | LL12NS01 |
| 75 | 13 | 57705 | LL13NS01 |
| 80 | 13 | 57757 | LL13NS02 |
| 85 | 14 | 57706 | LL14NS01 |
| 90 | 14/15 | 57707 | LL99NS01 |
| 95 | 15 | 57737 | LL15NS01 |
| 100 | 16 | 57758 | LL16NS03 |
| 105 | 17 | 57759 | LL17NS02 |
| 110 | 18 | 57710 | LL18NS01 |
| 115 | 19 | 57711 | LL19NS01 |
| 120 | 20 | 57712 | LL20NS01 |
| 125 | 21 | 57713 | LL21NS02 |
| 130 | 22 | 57714 | LL22NS01 |
| 135 | X | 57747 | LL0XNS01 |
| 140 | Y | 57715 | LL0YNS01 |

Alternatively, the extracted DNA from a sorted chromosome type can be amplified by the polymerase chain reaction (PCR) rather than cloning the extracted DNA in a vector or propagating it in a cell line. Appropriate tails are added to the extracted DNA in preparation for PCR. References for such PCR procedures are set out in Section I.B infra.

Other possible methods of isolating the desired sequences from hybrid cells include those of Schmeckpeper et al., "Partial Purification and Characterization of DNA from Human X Chromosome," *Proc. Natl. Acad. Sci.*, Vol. 76, pp. 6525-6528 (1979); or Olsen et al., *supra*(in Background).

2. Isolating DNA from a Portion of a Chromosome. Among the methods that can be used for isolating region-specific chromosomal DNA include the selection of an appropriate chromosomal region from DNA that has previously been mapped, for example, from a library of mapped cosmids; the sorting of derivative chromosomes, for example, by FACS; the microdissection of selected chromosomal material; subtractive hybridization; identification of an appropriate hybrid cell containing a desired chromosomal fragment, extracting and amplifying the DNA, and selecting the desired amplified DNA; and the selection of appropriate chromosomal material from radiation hybrids. The standard genetic engineering techniques outlined above in subsection I.A.1 are used in such procedures well-known to those in the art. Amplification of the region-specific DNA can be performed by cloning in an appropriate vector, propagating in an appropriate cell line, and/or by the use of PCR (see I.B infra).

A preferred method of isolating chromosomal region-specific DNA is to use mapped short DNA sequences to probe a library of longer DNA sequences, wherein the latter library has usually been cloned in a different vector. For example, a probe cloned in a plasmid can be used to probe a cosmid or yeast artificial chromosome (YAC) library. By using an initial seed probe, overlapping clones in the larger insert library can be found (a process called "walking"), and a higher complexity probe can be produced for reliable staining of the chromosomal region surrounding the seed probe. Ulti-

mately, when an entire genome for a species has been mapped (for example, by the Human Genome Project for the human species), ordered clones for the entire genome of the species will be available. One can then easily select the appropriate clones to form a probe of the desired specificity.

Another method of isolating DNA from a chromosomal region or regions (or also a whole chromosome) is to propagate such a chromosomal region or regions in an appropriate cell line (for example, a hybrid cell line such as a human/hamster hybrid cell), extract the DNA from the cell line and clone it in an appropriate vector and select clones containing human DNA to form a library. When a hybrid cell is used, the chromosomes in the hybrid cell containing the human chromosomal material may be separated by flow sorting (FACS) prior to Cloning to increase the frequency of human clones in the library. Still further, total DNA from the hybrid cell can be isolated and labeled without further cloning and used as a probe, as exemplified in Figure 6.

3. Single-Stranded Probes. In some cases, it is preferable that the nucleic acid fragments of the heterogeneous mixture consist of single-stranded RNA or DNA. Under some conditions, the binding efficiency of single-stranded nucleic acid probes has been found to be higher during *in situ* hybridization, e.g., Cox et al., "Detection of mRNAs in Sea Urchin Embryos by *In Situ* Hybridization Using Asymmetric RNA Probes," *Developmental Biology*, Vol. 101, pp. 485-502 (1984).

Standard methods are used to generate RNA fragments from isolated DNA fragments. For example, a method developed by Green et al., described in *Cell*, Vol. 32, pp. 681-694 (1983), is commercially available from Promega Biotec (Madison, WI) under the tradename "Riboprobe." Other transcription kits suitable for use with the present invention are available from United States Biochemical Corporation (Cleveland, OH) under the tradename "Genescribe." "Single-stranded DNA probes can be produced with the single-stranded bacteriophage M13, also available in kit form, e.g. Bethesda Research Laboratories (Gaithersburg, MD). The hybridizations illustrated in Figure 4 were performed with the libraries of Table 1 subcloned into the Bluescribe plasmid vector (Stratagene, La Jolla, CA). The Bluescribe plasmid contains RNA promoters which permit production of single-stranded probes.

Section IX, infra provides methods for preparing and applying non-self-complementary single-stranded nucleic acid probes that improve signal-to-noise ratios attainable in *in situ* hybridization by reducing non-specific and mismatched binding of the probe. That section further provides for methods of denaturing double-stranded target nucleic acid which minimizes single-stranded regions available for hybridization that are non-complementary to probe sequences. Briefly, probe is constructed by treating DNA with a restriction enzyme and an exonuclease to form template/primers for a DNA polymerase. The digested strand is resynthesized in the presence of labeled nucleoside triphosphate precursor, and the labeled single-stranded fragments are separated from the resynthesized fragments to form the probe. The target nucleic acid is treated with the same restriction enzyme used to construct the probe, and is treated with an exonuclease before application of the probe.

I.B. PCR

Another method of producing probes of this invention includes the use of the polymerase chain reaction [PCR]. [For an explanation of the mechanics of PCR, see Saiki et al., *Science*, 230: 1350 (1985) and U.S. Patent Nos. 4,683,195, 4,683,202 (both issued July 28, 1987) and 4,800,159 (issued January 24, 1989).] Target-specific nucleic acid sequences, isolated as indicated above, can be amplified by PCR to produce target-specific sequences which are reduced in or free of repetitive sequences. The PCR primers used for such a procedure are for the ends of the repetitive sequences, resulting in amplification of sequences flanked by the repeats.

Figure 7 illustrates such a method of using PCR wherein the representative repetitive sequence is Alu. If only short segments are amplified, it is probable that such sequences are free of other repeats, thus providing DNA reduced in repetitive sequences.

One can further suppress production of repetitive sequences in such a PCR procedure by first hybridizing complementary sequences to said repetitive sequence wherein said complementary sequences have extended non-complementary flanking ends or are terminated in nucleotides which do not permit extension by the polymerase. The non-complementary ends of the blocking sequences prevent the blocking sequences from acting as a PCR primer during the PCR process.

II. Removal of Repetitive Sequences and/or Disabling the Hybridization Capacity of Repetitive Sequences

Typically a probe of the current invention is produced in a number of steps including: obtaining source nucleic acid sequences that are complementary to the target region of the genome, labeling and otherwise processing them so that they will hybridize efficiently to the target and can be detected after they bind, and treating them to either disable the hybridization capacity or remove a sufficient proportion of shared repetitive sequences, or both disable and remove such sequences. The order of these steps depends on the specific procedures employed.

The following methods can be used to remove shared repetitive sequences and/or disable the hybridization ca-

pacity of such shared repetitive sequences. Such methods are representative and are expressed schematically in terms of procedures well known to those of ordinary skill the art, and which can be modified and extended according to parameters and procedures well known to those in the art.

5 1. Single-copy probes. A single-copy probe consists of nucleic acid fragments that are complementary to single-copy sequences contained in the target region of the genome. One method of constructing such a probe is to start with a DNA library produced by cloning the target region. Some of the clones in the library will contain DNA whose entire sequence is single-copy; others will contain repetitive sequences; and still others will have portions of single-copy and repetitive sequences. Selection, on a clone by clone basis, and pooling of those clones containing only single-copy sequences will result in a probe that will hybridize specifically to the target region. The single-copy nature of a clone can ultimately be established by Southern hybridization using standard techniques. Figure 4H shows hybridization with 10 120 clones selected in this way from a chromosome 4 library.

15 Southern analysis is very time consuming and labor intensive. Therefore, less perfect but more efficient screening methods for obtaining candidate single-copy clones are useful. In Section V.B, examples of improved methods are provided for screening individual phage and plasmid clones for the presence of repetitive DNA using hybridization with genomic DNA. The screening of plasmid clones is more efficient, and approximately 80% of selected clones contain only single-copy sequences; the remainder contain low-copy repeats. However, probes produced in this way can produce adequate staining contrast, indicating that the low-copy repetitive sequences can be tolerated in the probe (see subsection 3 of this section).

20 A disadvantage of clone by clone procedures is that a clone is discarded even if only a portion of the sequence it contains is repetitive. The larger the length of the cloned nucleic acid, the greater the chance that it will contain a repetitive sequence. Therefore, when nucleic acid is propagated in a vector that contains large inserts such as a cosmid, YAC, or in a cell line, such as hybrid cells, it may be advantageous to subclone it in smaller pieces before the single-copy selection is performed. The selection procedures just outlined above do not discriminate between shared and specific repetitive sequences; clones with detectable repetitive sequences of either type are not used in the probe.

25 2. Individual testing of hybridization properties. The hybridization specificity of a piece of nucleic acid, for example, a clone, can be tested by *in situ* hybridization. If under appropriate hybridization conditions it binds to single-copy or repetitive sequences specific for the desired target region, it can be included in the probe. Many sequences with specific hybridization characteristics are already known, such as chromosome-specific repetitive sequences [Trask et al., *supra*, (1988) and references therein], VNTRs, numerous mapped single copy sequences. More are continuously being mapped. Such sequences can be included in a probe of this invention.

30 3. Bulk Procedures. In many genomes, such as the human genome, a major portion of shared repetitive DNA is contained in a few families of highly repeated sequences such as Alu. A probe that is substantially free of such high-copy repetitive sequences will produce useful staining contrast in many applications. Such a probe can be produced from some source of nucleic acid sequences, for example, the libraries of Table I, with relatively simple bulk procedures. 35 Therefore, such bulk procedures are the preferred methods for such applications.

These methods primarily exploit the fact that the hybridization rate of complementary nucleic acid strands increases as their concentration increases. Thus, if a heterogeneous mixture of nucleic acid fragments is denatured and incubated under conditions that permit hybridization, the sequences present at high concentration will become double-stranded more rapidly than the others. The double-stranded nucleic acid can then be removed and the remainder used as a probe. Alternatively, the partially hybridized mixture can be used as the probe, the double-stranded sequences being unable to bind to the target. The following are methods representative of bulk procedures that are useful for producing the target-specific staining of this invention.

40 3a. Self-reassociation of the probe. Double-stranded probe nucleic acid in the hybridization mixture is denatured and then incubated under hybridization conditions for a time sufficient for the high-copy sequences in the probe to become substantially double-stranded. The hybridization mixture is then applied to the sample. The remaining labeled single-stranded copies of the highly repeated sequences bind throughout the sample producing a weak, widely distributed signal. The binding of the multiplicity of low-copy sequences specific for the target region of the genome produce an easily distinguishable specific signal.

45 Such a method is exemplified in Section VI.B (*infra*) with chromosome-specific libraries for chromosomes 4 and 21 (pBS4 and pBS21) as probes for those chromosomes. [Pinkel et al., *PNAs (USA)*, 85: 85:9138-9142 (December 1988)]. The hybridization mix, containing a probe concentration in the range of 1-10 ng/ μ l was heated to denature the probe and incubated at 37°C for 24 hours prior to application-to-the-sample.

50 3b. Use of blocking nucleic acid. Unlabeled nucleic acid sequences which are complementary to those sequences in the probe whose hybridization capacity it is desired to inhibit are added to the hybridization mixture. The probe and blocking nucleic acid are denatured, if necessary, and incubated under appropriate hybridization conditions. The sequences to be blocked become double-stranded more rapidly than the others, and therefore are unable to bind to the target when the hybridization mixture is applied to the target. In some cases, the blocking reaction occurs so quickly 55 that the incubation period can be very short; and adequate results can be obtained if the hybridization mix is applied

to the target immediately after denaturation. A blocking method is generally described by Sealy et al., "Removal of Repeat Sequences from Hybridization Probes", *Nucleic Acid Research*, 13: 1905 (1985). Examples of blocking nucleic acids include genomic DNA, a high-copy fraction of genomic DNA and particular sequences as outlined below (i-iii).

5 3b.i. Genomic DNA. Genomic DNA contains all of the nucleic acid sequences of the organism in proportion to their copy-number in the genome. Thus, adding genomic DNA to the hybridization mixture increases the concentration of the high-copy repeat sequences more than low-copy sequences, and therefore is more effective at blocking the former. However, the genomic DNA does contain copies of the sequences that are specific to the target and so will also reduce the desired chromosome-specific binding if too much is added. Guidelines to determine how much genomic DNA to add (see 3.e. Concept of Q, infra) and examples of using genomic blocking DNA are provided below. The blocking effectiveness of genomic DNA can be enhanced under some conditions by adjusting the timing of its addition to the hybridization mix; examples of such timing adjustments are provided with Protocol I and Protocol II hybridizations illustrated in Figures 4B through E (Protocol I) and Figure 4F (Protocol II) and detailed in Section VI, *infra*.

10 15 3b.ii. High-copy fraction of genomic DNA. The difficulty with use of genomic DNA is that it also blocks the hybridization of the low-copy sequences, which are predominantly the sequences that give the desired target staining. Thus, fractionating the genomic DNA to obtain only the high-copy sequences and using them for blocking overcomes this difficulty. Such fractionation can be done, for example, with hydroxyapatite as described below (3c.i).

20 25 3b.iii. Specified sequences. The blocking of a particular sequence in the probe can be accomplished by adding many unlabeled copies of that sequence. For example, Alu sequences in the probe can be blocked by adding cloned Alu DNA. Blocking DNA made from a mixture of a few clones containing the highest copy sequences in the human genome can be used effectively with chromosome-specific libraries for example, those of Table I. Alternatively, unlabeled nucleic acid sequences from one or more chromosome-specific libraries could be used to block a probe containing labeled sequences from one or more other chromosome-specific libraries. The shared sequences would be blocked whereas sequences occurring only on the target chromosome would be unaffected. Figure 4F shows that genomic DNA was not effective in completely blocking the hybridization of a sequence or sequences shared by human chromosome 21 and the centromeric regions of the other human acrocentric chromosomes. When a clone or clones containing such a sequence or sequences is or are eventually isolated, unlabeled DNA produced therefrom could be added to the genomic blocking DNA to improve the specificity of the staining.

3c. Removal of Sequences.

30 3c.i. Hydroxyapatite. Single- and double-stranded nucleic acids have different binding characteristics to hydroxyapatite. Such characteristics provide a basis commonly used for fractionating nucleic acids. Hydroxyapatite is commercially available (eg. Bio-Rad Laboratories, Richmond, CA). The fraction of genomic DNA containing sequences with a particular degree of repetition, from the highest copy-number to single-copy, can be obtained by denaturing genomic DNA, allowing it to reassociate under appropriate conditions to a particular value of C_0t , followed by separation using hydroxyapatite. The single- and double-stranded nucleic acid can also be discriminated by use of S1 nuclease. Such techniques and the concept of C_0t are explained in Britten et al., "Analysis of Repeating DNA Sequences by Reassociation", in Methods in Enzymology, Vol. 29, pp. 363-418 (1974).

40 45 The single-stranded nucleic acid fraction produced in 3a. or 3b. above can be separated by hydroxyapatite and used as a probe. Thus, the sequences that have been blocked (that become double-stranded) are physically removed. The probe can then be stored until needed. The probe can then be used without additional blocking nucleic acid, or its staining contrast can perhaps be improved by additional blocking.

50 55 3c.ii. Reaction with immobilized nucleic acid. Removal of particular sequences can also be accomplished by attaching single-stranded "absorbing" nucleic acid sequences to a solid support. Single-stranded source nucleic acid is hybridized to the immobilized nucleic acid. After the hybridization, the unbound sequences are collected and used as the probe. For example, human genomic DNA can be used to absorb repetitive sequences from human probes. One such method is described by Brison et al., "General Method for Cloning Amplified DNA by Differential Screening with Genomic Probes," *Molecular and Cellular Biology*, Vol. 2, pp. 578-587 (1982). Briefly, minimally sheared human genomic DNA is bound to diazonium cellulose or a like support. The source DNA, appropriately cut into fragments, is hybridized against the immobilized DNA to C_0t values in the range of about 1 to 100. The preferred stringency of the hybridization conditions may vary depending on the base composition of the DNA. Such a procedure could remove repetitive sequences from chromosome-specific libraries, for example, those of Table I, to produce a probe capable of staining a whole human chromosome.

3d. Blocking non-targeted sequences in the targeted genome. Blocking of non-targeted binding sites in the targeted genome by hybridization with unlabeled complementary sequences will prevent binding of labeled sequences in the probe that have the potential to bind to those sites. For example, hybridization with unlabeled genomic DNA will render the high-copy repetitive sequences in the target genome double-stranded. Labeled copies of such sequences in the probe will not be able to bind when the probe is subsequently applied.

In practice, several mechanisms combine to produce the staining contrast. For example, when blocking DNA is added to the probe as in 3b above, that which remains single-stranded when the probe is applied to the target can bind to and block the target sequences. If the incubation of the probe with the blocking DNA is minimal, then the genomic DNA simultaneously blocks the probe and competes with the probe for binding sites in the target.

5 3e. Concept of Q. As mentioned in section 3b.i above, it is necessary to add the correct amount of genomic DNA to achieve the best compromise between inhibiting the hybridization capacity of high-copy repeats in the probe and reducing the desired signal intensity by inhibition of the binding of the target-specific sequences. The following discussion pertains to use of genomic blocking DNA with probes produced by cloning or otherwise replicating stretches of DNA from the target region of the genome. Thus, the probe contains a representative sampling of the single-copy, 10 chromosome-specific repetitive sequences, and shared repetitive sequences found in the target. Such a probe might range in complexity from 100 kb of sequence derived from a small region of the genome, for example several closely spaced cosmid clones; to many millions of bases, for example a combination of multiple libraries from Table I. The discussion below is illustrative and can be extended to other situations where different blocking nucleic acids are used. The following discussion of Q is designed only to give general guidelines as to how to proceed.

15 The addition of unlabeled genomic DNA to a hybridization mix containing labeled probe sequences increases the concentration of all of the sequences, but increases the concentration of the shared sequences by a larger factor than the concentration of the target-specific sequences because the shared sequences are found elsewhere in the genome, whereas the target-specific sequences are not. Thus, the reassociation of the shared sequences is preferentially enhanced so that the hybridization of the labeled copies of the shared sequences to the target is preferentially inhibited.

20 To quantify this concept, first consider one of the sequences, repeat or single-copy, that hybridize specifically to the i th chromosome in a hybridization mixture containing a mass m_p of probe DNA from the i th chromosome library of Table 1 (for example) and m_b of unlabeled genomic DNA. The number of labeled copies of the sequence is proportional to m_p . However, the number of unlabeled copies is proportional to $f_i m_b$, where f_i is the fraction of genomic DNA contained 25 on the i th chromosome. Thus, the ratio of unlabeled to labeled copies of each of the sequences specific for the target chromosome, is $f_i m_b/m_p$, which is defined herein as Q. For normal human chromosomes, $0.016 \leq f_i \leq 0.08$ [Mendelsohn et al., Science, 179: 1126 (1973)]. For representative examples described in Section VI.B (infra), $f_4 = 0.066$ and $f_{21} = 0.016$. For a probe targeted at a region comprised of L base pairs, $f_i = L/G$ where G is the number of base pairs in a genome (approximately 3×10^9 bases for humans and other mammals). Thus, $Q = (L/G)(m_b/m_p)$.

Now consider a shared sequence that is distributed more-or-less uniformly over the genome, for example, Alu. 30 The number of labeled copies is proportional to m_p , whereas the number of unlabeled copies is proportional to m_b . Thus, the ratio of unlabeled to labeled copies is $m_b/m_p = Q/f_i$. This is true for all uniformly distributed sequences, regardless of copy number. Thus adding genomic DNA increases the concentration of each specific sequence by the factor $1+Q$, whereas each uniformly distributed sequence is increased by the larger factor $1+Q/f_i$. Thus, the reassociation rates of the shared sequences are increased by a larger factor than those of the specific sequences by the addition 35 of genomic DNA.

It can be shown that roughly half of the beneficial effect of genomic DNA on relative reassociation rates is achieved when $Q=1$, and, by $Q=5$, there is essentially no more benefit to be gained by further increases. Thus, the protocol 1 hybridizations of Section VI.B infra keep $Q \leq 5$.

To illustrate the use of genomic blocking DNA, it is convenient to consider a model of a genome wherein 50% of 40 the DNA is comprised of specific sequences (both repetitive and single-copy) and the other 50% of the DNA is comprised of shared repetitive sequences that are distributed uniformly over the genome. Thus, according to the model, if the target is L bases (that is, the probe contains fragments representing L bases of the target area or areas of the genome), sequences containing $L/2$ bases will be specific to the target, and $L/2$ will be shared with the entire genome.

45 III. Labeling the Nucleic Acid Fragments of the Heterogeneous Mixture.

Several techniques are available for labeling single- and double-stranded nucleic acid fragments of the heterogeneous mixture. They include incorporation of radioactive labels, e.g. Harper et al., Chromosoma, Vol 83, pp. 431-439 50 (1984); direct attachment of fluorochromes or enzymes, e.g. Smith et al., Nucleic Acids Research, Vol. 13, pp. 2399-2412 (1985), and Connolly et al., Nucleic Acids Research, Vol. 13, pp. 4485-4502 (1985); and various chemical modifications of the nucleic acid fragments that render them detectable immunochemically or by other affinity reactions, e.g. Tchen et al., "Chemically-Modified-Nucleic Acids as Immunodetectable Probes in-Hybridization Experiments," Proc. Natl. Acad. Sci., Vol 81, pp. 3466-3470 (1984); Richardson et al., "Biotin and Fluorescent Labeling of RNA Using T4 RNA Ligase," Nucleic Acids Research, Vol. 11, pp. 6167-6184 (1983); Langer et al., "Enzymatic Synthesis of Biotin-55 Labeled Polynucleotides: Novel Nucleic Acid Affinity Probes," Proc. Natl. Acad. Sci., Vol. 78, pp. 6633-6637 (1981); Brigati et al., "Detection of Viral Genomes in Cultured Cells and Paraffin-Embedded Tissue Sections Using Biotin-Labeled Hybridization Probes," Virology, Vol. 126, pp. 32-50 (1983); Broker et al., "Electron Microscopic Visualization of tRNA Genes with Ferritin-Avidin: Biotin Labels," Nucleic Acids Research, Vol. 5, pp. 363-384 (1978); Bayer et al.,

"The Use of the Avidin Biotin Complex as a Tool in Molecular Biology," Methods of Biochemical Analysis, Vol. 26, pp. 1-45 (1980) Kuhlmann, Immunoenzyme Techniques in Cytochemistry (Weinheim, Basel, 1984). Langer-Safer et al., PNAS USA, 79: 4381 (1982); Landegent et al., Exp. Cell Res., 153: 61 (1984); and Hopman et al., Exp. Cell Res., 169: 357 (1987).

5 Exemplary labeling means include those wherein the probe fragments are biotinylated, modified with N-acetoxy-N-2-acetylaminofluorene, modified with fluorescein isothiocyanate, modified with mercury/TNP ligand, sulfonated, digoxigenated or contain T-T dimers.

10 The key feature of "probe labeling" is that the probe bound to the target be detectable. In some cases, an intrinsic feature of the probe nucleic acid, rather than an added feature, can be exploited for this purpose. For example, antibodies that specifically recognize RNA/DNA duplexes have been demonstrated to have the ability to recognize probes made from RNA that are bound to DNA targets [Rudkin and Stollar, Nature, 265: 472-473 (1977)]. The RNA used for such probes is unmodified. Probe nucleic acid fragments can be extended by adding "tails" of modified nucleotides or particular normal nucleotides. When a normal nucleotide tail is used, a second hybridization with nucleic acid complementary to the tail and containing fluorochromes, enzymes, radioactivity, modified bases, among other labeling means, allows detection of the bound probe. Such a system is commercially available from Enzo Biochem (Biobridge Labeling System; Enzo Biochem Inc., New York, N.Y.).

15 Another example of a means to visualize the bound probe wherein the nucleic acid sequences in the probe do not directly carry some modified constituent is the use of antibodies to thymidine dimers. Nakane et al., 20 (2): 229 (1987), illustrate such a method wherein thymine-thymine dimerized DNA (T-T DNA) was used as a marker for *in situ* hybridization. The hybridized T-T DNA was detected immunohistochemically using rabbit anti-T-T DNA antibody.

20 All of the labeling techniques disclosed in the above references may be preferred under particular circumstances. Accordingly, the above-cited references are incorporated by reference. Further, any labeling techniques known to those in the art would be useful to label the staining compositions of this invention. Several factors govern the choice of labeling means, including the effect of the label on the rate of hybridization and binding of the nucleic acid fragments to the chromosomal DNA, the accessibility of the bound probe to labeling moieties applied after initial hybridization, the mutual compatibility of the labeling moieties, the nature and intensity of the signal generated by the label, the expense and ease in which the label is applied, and the like.

25 Several different high complexity probes, each labeled by a different method, can be used simultaneously. The binding of different probes can thereby be distinguished, for example, by different colors.

30

IV. In Situ Hybridization.

Application of the heterogeneous mixture of the invention to chromosomes is accomplished by standard *in situ* hybridization techniques. Several excellent guides to the technique are available, e.g., Gall and Pardue, "Nucleic Acid Hybridization in Cytological Preparations," Methods in Enzymology, Vol. 21, pp. 470-480 (1981); Henderson, "Cytological Hybridization to Mammalian Chromosomes," International Review of Cytology, Vol. 76, pp. 1-46 (1982); and Angerer, et al., "In Situ Hybridization to Cellular RNAs," in Genetic Engineering: Principles and Methods, Setlow and Hollaender, Eds., Vol. 7, pp. 43-65 (Plenum Press, New York, 1985).

35 Three factors influence the staining sensitivity of the hybridization probes: (1) efficiency of hybridization (fraction of target DNA that can be hybridized by probe), (2) detection efficiency (i.e., the amount of visible signal that can be obtained from a given amount of hybridization probe), and (3) level of noise produced by nonspecific binding of probe or components of the detection system.

40 Generally *in situ* hybridization comprises the following major steps: (1) fixation of tissue or biological structure to be examined, (2) prehybridization treatment of the biological structure to increase accessibility of target DNA, and to reduce nonspecific binding, (3) hybridization of the heterogeneous mixture of probe to the DNA in the biological structure or tissue; (4) posthybridization washes to remove probe not bound in specific hybrids, and (5) detection of the hybridized probes of the heterogeneous mixture. The reagents used in each of these steps and their conditions of use vary depending on the particular situation.

45 The following comments are meant to serve as a guide for applying the general steps listed above. Some experimentation may be required to establish optimal staining conditions for particular applications.

50 In preparation for the hybridization, the probe, regardless of the method of its production, may be broken into fragments of the size appropriate to obtain the best intensity-and-specificity-of-hybridization. As a general guideline concerning the size of the fragments, one needs to recognize that if the fragments are too long they are not able to penetrate into the target for binding and instead form aggregates that contribute background noise to the hybridization; however, if the fragments are too short, the signal intensity is reduced.

55 Under the conditions of hybridization exemplified in Section VI.B wherein human genomic DNA is used as an agent to block the hybridization capacity of the high copy shared repetitive sequences, the preferred size range of the probe fragments is from about 200 bases to about 2000 bases, more preferably in the vicinity of 1 kb. When the size of the

probe fragments is in about the 800 to about 1000 base range, the preferred hybridization temperature is about 30°C to about 45°C, more preferably about 35°C to about 40°C, and still more preferably about 37°C; preferred washing temperature range is from about 40°C to about 50°C, more preferably about 45°C.

The size of the probe fragments is checked before hybridization to the target; preferably the size of the fragments is monitored by electrophoresis, more preferably by denaturing agarose gel electrophoresis.

Fixatives include acid alcohol solutions, acid acetone solutions, Petrunkevitsch's reagent, and various aldehydes such as formaldehyde, paraformaldehyde, glutaraldehyde, or the like. Preferably, ethanol-acetic acid or methanol-acetic acid solutions in about 3:1 proportions are used to fix the chromosomes in metaphase spreads. For cells or chromosomes in suspension, a fixation procedure disclosed by Trask, et al., in Science, Vol. 230, pp. 1401-1402 (1985), is useful. Accordingly, Trask et al., is incorporated by reference. Briefly, K₂CO₃ and dimethylsulfoxide (DMS) are added (from a 5x concentrated stock solution, mixed immediately before use) to a suspension containing about 5x10⁶ nuclei/ml. Final K₂CO₃ and DMS concentrations are 20 mM and 3 mM, respectively. After 15 minutes at 25°C, the pH is adjusted from 10.0 to 8.0 by the addition of 50 microliters of 100 mM citric acid per milliliter of suspension. Nuclei are washed once by centrifugation (300g, 10 minutes, 4°C in 50 mM KCl, 5 mM Hepes buffer, at pH 9.0, and 10 mM MgSO₄).

A preferred fixation procedure for cells or nuclei in suspension is disclosed by Trask et al., Hum. Genet., 78: 251-259 (1988). Briefly, nuclei are fixed for about 10 minutes at room temperature in 1% paraformaldehyde in PBS, 50 mM MgSO₄, pH 7.6 and washed twice. Nuclei are resuspended in isolation buffer (IB) (50 mM KC1, 5 mM HEPES, 10 mM MgSO₄, 3 mM dithioerythritol, 0.15 mg/ml RNase, pH 8.0)/0.05% Triton X-100 at 10⁸/ml.

Frequently before *in situ* hybridization chromosomes are treated with agents to remove proteins. Such agents include enzymes or mild acids. Pronase, pepsin or proteinase K are frequently used enzymes. A representative acid treatment is 0.02-0.2 N HCl, followed by high temperature (e.g., 70°C) washes. Optimization of deproteinization requires a combination of protease concentration and digestion time that maximizes hybridization, but does not cause unacceptable loss of morphological detail. Optimum conditions vary according to tissue types and method of fixation. Additional fixation after protease treatment may be useful. Thus, for particular applications, some experimentation may be required to optimize protease treatment.

In some cases pretreatment with RNase may be desirable to remove residual RNA from the target. Such removal can be accomplished by incubation of the fixed chromosomes in 50-100 microgram/milliliter RNase in 2X SSC (where SSC is a solution of 0.15 M NaCl and 0.015M sodium citrate) for a period of 1-2 hours at room temperature.

The step of hybridizing the probes of the heterogeneous probe mixture to the chromosomal DNA involves (1) denaturing the target DNA so that probes can gain access to complementary single-stranded regions, and (2) applying the heterogeneous mixture under conditions which allow the probes to anneal to complementary sites in the target. Methods for denaturation include incubation in the presence of high pH, low pH, high temperature, or organic solvents such as formamide, tetraalkylammonium halides, or the like, at various combinations of concentration and temperature. Single-stranded DNA in the target can also be produced with enzymes, such as, Exonuclease III [van Dekken et al., Chromosoma (Berl) 97: 1-5 (1988)]. The preferred denaturing procedure is incubation for between about 1-10 minutes in formamide at a concentration between about 35-95 percent in 2X SSC and at a temperature between about 25-70°C. Determination of the optimal incubation time, concentration, and temperature within these ranges depends on several variables, including the method of fixation and type of probe nucleic acid (for example, DNA or RNA).

After the chromosomal DNA is denatured, the denaturing agents are typically removed before application of the heterogeneous probe mixture. Where formamide and heat are the primary denaturing agents, removal is conveniently accomplished by several washes with a solvent, which solvent is frequently chilled, such as a 70%, 85%, 100% cold ethanol series. Alternatively the composition of the denaturant can be adjusted as appropriate for the *in situ* hybridization by addition of other constituents or washes in appropriate solutions. The probe and target nucleic acid may be denatured simultaneously by applying the hybridization mixture and then heating to the appropriate temperature.

The ambient physicochemical conditions of the chromosomal DNA and probe during the time the heterogeneous mixture is applied is referred to herein as the hybridization conditions, or annealing conditions. Optimal hybridization conditions for particular applications can be adjusted by controlling several factors, including concentration of the constituents, incubation time of chromosomes in the heterogeneous mixture, and the concentrations, complexities, and lengths of the nucleic acid fragments making up the heterogeneous mixture. Roughly, the hybridization conditions must be sufficiently close to the melting temperature to minimize nonspecific binding. On the other hand, the conditions cannot be so stringent as to reduce correct hybridizations of complementary sequences below detectable levels or to require excessively long incubation times.

The concentrations of nucleic acid in the hybridization mixture is an important variable. The concentrations must be high enough so that sufficient hybridization of respective chromosomal binding sites occurs in a reasonable time (e.g., within hours to several days). Higher concentrations than that necessary to achieve adequate signals should be avoided so that nonspecific binding is minimized. An important practical constraint on the concentration of nucleic acid in the probe in the heterogeneous mixture is solubility. Upper bounds exist with respect to the fragment concentration,

i.e., unit length of nucleic acid per unit volume, that can be maintained in solution and hybridize effectively.

In the representational examples described in Section VI.B (*infra*), the total DNA concentration in the hybridization mixture had an upper limit on the order of 1 ug/*ul*. Probe concentrations in the range of 1-20 ng/*ul* were used for such whole chromosome staining. The amount of genomic blocking DNA was adjusted such that Q was less than 5. At the low end of probe concentration, adequate signals were obtained with a one hour incubation, that is, a time period wherein the probe and blocking DNA are maintained together before application to the targeted material, to block the high-copy sequences and a 16 hour hybridization. Signals were visible after two hours of hybridization. The best results (bright signals with highest contrast) occurred after a 100 hour hybridization, which gave the low-copy target-specific sequences more opportunity to find binding sites. At the high end of the probe concentration, bright signals are obtained after hybridizations of 16 hours or less; the contrast was reduced since more labeled repetitive sequences were included in the probe.

The fixed target object can be treated in several ways either during or after the hybridization step to reduce non-specific binding of probe DNA. Such treatments include adding nonprobe, or "carrier", DNA to the heterogeneous mixture, using coating solutions, such as Denhardt's solution (*Biochem. Biophys. Res. Commun.*, Vol. 23, pp. 641-645 (1966), with the heterogeneous mixture, incubating for several minutes, e.g., 5-20, in denaturing solvents at a temperature 5-10°C above the hybridization temperature, and in the case of RNA probes, mild treatment with single strand RNase (e.g., 5-10 micrograms per millileter RNase) in 2X SSC at room temperature for 1 hour).

V. Chromosome-Specific Staining Reagents Comprising Selected Single-Copy Sequences

V.A. Making and Using a Staining Reagent Specific to Human Chromosome 21

V. A. 1. Isolation of Chromosome 21 and Construction of a Chromosome 21-Specific Library

DNA fragments from human chromosome-specific libraries are available from the National Laboratory Gene Library Project through the American Type Culture Collection (ATCC), Rockville, MD. DNA fragments from chromosome 21 were generated by the procedure described by Fuscoe et al., in "Construction of Fifteen Human Chromosome-Specific DNA Libraries from Flow-Purified Chromosomes," *Cytogenet. Cell Genet.*, Vol. 43, pp. 79-86 (1986). Briefly, a human diploid fibroblast culture was established from newborn foreskin tissue. Chromosomes of the cells were isolated by the MgSO₄ method of van den Engh et al., *Cytometry*, Vol. 5, pp. 108-123 (1984), and stained with the fluorescent dyes--Hoechst 33258 and Chromomycin A3. Chromosome 21 was purified on the Lawrence Livermore National Laboratory high speed sorter, described by Peters et al., *Cytometry*, Vol. 6, pp. 290-301 (1985).

After sorting, chromosome concentrations were approximately 4x10⁵/ml. Therefore, prior to DNA extraction, the chromosomes (0.2-1.0x10⁶) were concentrated by centrifugation at 40,000 x g for 30 minutes at 4°C. The pellet was then resuspended in 100 microliters of DNA isolation buffer (15 mM NaCl, 10 mM EDTA, 10 mM Tris HCl pH 8.0) containing 0.5% SDS and 100 micrograms/ml proteinase K. After overnight incubation at 37°C, the proteins were extracted twice with phenol: phenol:chloroform: isoamyl alcohol (25: 24: 1) and once with chloroform:isoamyl isoamyl alcohol (24: 1). Because of the small amounts of DNA, each organic phase was reextracted with a small amount of 10 mM Tris pH 8.0, 1 mM EDTA (TE). Aqueous layers were combined and transferred to a Schleicher and Schuell minicollodion membrane (#UHO20/25) and dialyzed at room temperature against TE for 6-8 hours. The purified DNA solution was then digested with 50 units of HindIII (Bethesda Research Laboratories, Inc.) in 50 mM NaCl, 10 mM Tris HCl pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol. After 4 hours at 37°, the reaction was stopped by extractions with phenol and chloroform as described above. The aqueous phase was dialyzed against water overnight at 4°C in a minicollodion bag and then 2 micrograms of Charon 21A arms cleaved with HindIII and treated with calf alkaline phosphatase (Boehringer Mannheim) were added. This solution was concentrated under vacuum to a volume of 50-100 microliters and transferred to a 0.5 ml microfuge tube where the DNA was precipitated with one-tenth volume 3M sodium acetate pH 5.0 and 2 volumes ethanol. The precipitate was collected by centrifugation, washed with cold 70% ethanol, and dissolved in 10 microliters of TE.

After allowing several hours for the DNA to dissolve, 1 microliter of 10X ligase buffer (0.5M Tris HCl pH 7.4, 0.1 M MgCl₂, 0.1M dithiothreitol, 10 mM ATP, 1 mg/ml bovine serum albumin) and 1 unit of T4 ligase (Bethesda Research Laboratory, Inc.) were added. The ligation reaction was incubated at 10°C for 16-20 hours and 3 microliter aliquots were packaged-into-phage-particles using *in vitro*-extracts prepared from *E. coli*-strains BHB-2688 and BHB-2690, described by Hohn in *Methods in Enzymology*, Vol. 68, pp. 299-309 (1979) *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory, New York, 1982). Briefly, both extracts were prepared by sonication and combined at the time of *in vivo* packaging. These extracts packaged wild-type lambda DNA at an efficiency of 1-5x10⁸ plaque forming units (pfu) per microgram. The resultant phage were amplified on *E. coli* LE392 at a density of approximately 10⁴ pfu/150 mm dish for 8 hours to prevent plaques from growing together and to minimize differences in growth rates of different recombinants. The phage were eluted from the agar in 10 ml SM buffer (50 mM Tris HCl pH 7.5, 10 mM

MgSO₄, 100 mM NaCl, 0.01% gelatin) per plate by gentle shaking at 4°C for 12 hours. The plates were then rinsed with an additional 4 ml of SM. After pelleting cellular debris, the phage suspension was stored over chloroform at 4°C.

V.A.2. Construction and Use of Chromosome 21- Specific Stain for Staining Chromosome 21 of Human Lymphocytes

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Clones having unique sequence inserts are isolated by the method of Benton and Davis, Science, Vol. 196, pp. 180-182 (1977). Briefly, about 1000 recombinant phage are isolated at random from the chromosome 21-specific library. These are transferred to nitrocellulose and probed with nick translated total genomic human DNA.

10 Of the clones which do not show strong hybridization, approximately 300 are picked which contain apparent unique sequence DNA. After the selected clones are amplified, the chromosome 21 insert in each clone is ³²P labeled and hybridized to Southern blots of human genomic DNA digested with the same enzyme used to construct the chromosome 21 library, i.e., Hind III. Unique sequence containing clones are recognized as those that produce a single band during Southern analysis. Roughly, 100 such clones are selected for the heterogeneous mixture. The unique sequence clones are amplified, the inserts are removed by Hind III digestions, and the inserts are separated from the phage arms by 15 gel electrophoresis. The probe DNA fragments (i.e., the unique sequence inserts) are removed from the gel and biotinylated by nick translation (e.g., by a kit available from Bethesda Research Laboratories). Labeled DNA fragments are separated from the nick translation reaction using small spin columns made in 0.5 ml Eppendorph tubes filled with Sephadex® G-50 (medium) swollen in 50 mM Tris, 1 mM EDTA, 0.1% SDS, at pH 7.5. Human lymphocyte chromosomes are prepared following Harper et al, Proc. Natl. Acad. Sci., Vol. 78, pp. 4458-4460 (1981). Metaphase and 20 interphase cells were washed 3 times in phosphate buffered saline, fixed in methanol-acetic acid (3: 1) and dropped onto cleaned microscope slides. Slides are stored in a nitrogen atmosphere at-20°C.

Slides carrying interphase cells and/or metaphase spreads are removed from the nitrogen, heated to 65°C for 4 hours in air, treated with RNase (100 micrograms/ml for 1 hour at 37°C), and dehydrated in an ethanol series. They are then treated with proteinase K (60 ng/ml at 37°C for 7.5 minutes) and dehydrated. The proteinase K concentration is adjusted depending on the cell type and enzyme lot so that almost no phase microscopic image of the chromosomes remains on the dry slide. The hybridization mix consists of (final concentrations) 50 percent formamide, 2X SSC, 10 percent dextran sulfate, 500 micrograms/ml carrier DNA (sonicated herring sperm DNA), and 2.0 microgram/ml biotin-labeled chromosome 21-specific DNA. This mixture is applied to the slides at a density of 3 microliters/cm² under a glass coverslip and sealed with rubber cement. After overnight incubation at 37°C, the slides are washed at 45°C (50% formamide-2XSSC pH 7, 3 times 3 minutes; followed by 2XSSC pH 7, 5 times 2 minutes) and immersed in BN buffer (0.1 M Na bicarbonate, 0.05 percent NP-40, pH 8). The slides are never allowed to dry after this point.

The slides are removed from the BN buffer and blocked for 5 minutes at room temperature with BN buffer containing 5% non-fat dry milk (Carnation) and 0.02% Na Azide (5 microliter/cm² under plastic coverslips). The coverslips are removed, and excess liquid briefly drained and fluorescein-avidin DCS (3 microgram/ml in BN buffer with 5% milk and 0.02% NaAzide) is applied (5 microliter/cm²). The same coverslips are replaced and the slides incubated 20 minutes at 37°C. The slides are then washed 3 times for 2 minutes each in BN buffer at 45°C. The intensity of biotin-linked fluorescence is amplified by adding a layer of biotinylated goat anti-avidin antibody (5 microgram/ml in BN buffer with 5% goat serum and 0.02% Na Azide), followed, after washing as above, by another layer of fluorescein-avidin DCS. Fluorescein-avidin DCS, goat antiavidin and goat serum are all available commercially, e.g., Vector Laboratories (Burlingame, CA). After washing in BN, a fluorescence antifade solution, p-phenylenediamine (1.5 microliter/cm² of coverslip) is added before observation. It is important to keep this layer thin for optimum microscopic imaging. This antifade significantly reduced fluorescein fading and allows continuous microscopic observation for up to 5 minutes. The DNA counterstains (DAPI or propidium iodide) are included in the antifade at 0.25-0.5 microgram/ml.

45 The red-fluorescing DNA-specific dye propidium iodide (PI) is used to allow simultaneous observation of hybridized probe and total DNA. The fluorescein and PI are excited at 450-490 nm (Zeiss filter combination 487709). Increasing the excitation wavelength to 546 nm (Zeiss filter combination 487715) allows observation of the PI only. DAPI, a blue fluorescent DNA-specific stain excited in the ultraviolet (Zeiss filter combination 487701), is used as the counterstain when biotin-labeled and total DNA are observed separately. Metaphase chromosome 21s are detected by randomly located spots of yellow distributed over the body of the chromosome.

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V. B. Improved Method for Efficiently Selecting Chromosome 21 Single-Copy Sequences

Fuscoe et al., Genomics, 5:100-109 (1989) provides more efficient procedures than the method described immediately above (V.A.2) for selecting large numbers of single-copy sequence or very low copy number repeat sequence clones from recombinant phage libraries and demonstrates their use to stain chromosome 21. Said article is hereby incorporated by reference. Briefly, clones were selected from the Charon 21A library LL21NS02 (made from DNA from human chromosome 21) using two basic procedures. In the first, the phage library was screened in two stages using methods designed to be more sensitive to the presence of repetitive sequences in the clones than the method of

Section V.A.2. The selected clones were then subcloned into plasmids. The 450 inserts thus selected form the library pBS-U21. The second was in a multistep process in which: 1) Inserts from LL21NS02 were subcloned into Bluescribe plasmids, 2) plasmids were grown at high density in bacterial colonies on nitrocellulose filters and 3) radioactive human genomic DNA was hybridized to the plasmid DNA on nitrocellulose filters at low stringency in two steps and 4) plasmids having inserts that failed to hybridize were selected as potentially carrying single-copy sequences. Fifteen hundred and thirty colonies were picked in this manner to form the library pBS-U21/1530.

Southern analysis indicated that the second procedure was more effective at recognizing repetitive sequence than the first. Fluorescence *in situ* hybridization with DNA from pBS-U21/1530 allowed specific, intense staining of the number 21 chromosomes in metaphase spreads made from human lymphocytes. Hybridization with pBS-U21 gives less specific staining of chromosome 21. Details concerning the Fuscoe et al. method of selecting single-copy sequence or very low repeat sequence probes from recombinant libraries can be found in Fuscoe et al., id.

V.C. Hybridization with a Collection of Chromosome 4 Single-Copy Sequences

Pinkel et al., PNAS (USA), 85: 9138-9142 (December 1988) describe the procedures for preparing chromosome 4 single-copy sequences and then a protocol [modification of the procedure described in Pinkel et al; PNAS (USA), 83:2934-2938 (1986)] for hybridizing said single copy probes to a human metaphase spread. Figure 4H shows the hybridization with a pool of 120 single-copy probes from chromosome 4 to a human metaphase spread.

VI. Incapacitating Shared Repetitive Sequences

VI.A. Chromosome 21-Specific Staining Using Blocking DNA

High concentrations of unlabeled human genomic DNA and lambda phage DNA were used to inhibit the binding of repetitive and vector DNA sequences to the target chromosomes. Heavy proteinase digestion and subsequent fixation of the target improved access of probes to target DNA.

Human metaphase spreads were prepared on microscope slides with standard techniques and stored immediately in a nitrogen atmosphere at -20°C.

Slides were removed from the freezer and allowed to warm to room temperature in a nitrogen atmosphere before beginning the staining procedure. The warmed slides were first treated with 0.6 microgram/ml proteinase K in P buffer (20 mM Tris, 2 mM CaCl₂ at pH 7.5) for 7.5 minutes, and washed once in P buffer. The amount of proteinase K used needs to be adjusted for different batches of slides. After denaturing the slides were stored in 2XSSC. A hybridization mix was prepared which consisted of 50% formamide, 10% dextran sulfate, 1% Tween 20, 2XSSC, 0.5 mg/ml human genomic DNA, 0.03 mg/ml lambda DNA, and 3 microgram/ml biotin labeled probe DNA. The probe DNA consisted of the highest density fraction of phage from the chromosome 21 Hind III fragment library(ATCC accession number 57713), as determined by a cesium chloride gradient.(Both insert and phage DNA of the probe were labeled by nick translation.) The average insert size (amount of chromosome 21 DNA), as determined by gel electrophoresis was about 5 kilobases. No attempt was made to remove repetitive sequences from the inserts or to isolate the inserts from the lambda phage vector. The hybridization mix was denatured by heating to 70°C for 5 minutes followed by incubation at 37°C for 1 hour. The incubation allows the human genomic DNA and unlabeled lambda DNA in the hybridization mix to block the human repetitive sequences and vector sequences in the probe.

The slide containing the human metaphase spread was removed from the 2XSSC and blotted dry with lens paper. The hybridization mix was immediately applied to the slide, a glass cover slip was placed on the slide with rubber cement, and the slide was incubated overnight at 37°C. Afterwards preparation of the slides proceeded as described in Section V.B. (wherein chromosome 21 DNA was stained with fluorescein and total chromosomal DNA counterstained with DAPI). Figures 1 A-C illustrate the results. Figure 1A is a DPAI image of the human metaphase spread obtained with a computerized image analysis system. It is a binary image showing everything above threshold as white, and the rest as black. The primary data was recorded as a gray level image with 256 intensity levels. (Small arrows indicate the locations of the chromosome 21s.) Figure 1B is a fluorescein image of the same spread as in Figure 1A, again in binary form. (Again, small arrows indicate the locations of the chromosome 21 s.) Figure 1 C illustrates the positions of the chromosome 21s after other less densely stained objects were removed by standard image processing techniques.

VI.B. Detection of Trisomy 21 and Translocations of Chromosome 4 Using Bluescribe Plasmid Libraries

As illustrated in Section VI.A., a human chromosome-specific library, including its shared repetitive sequences, can be used to stain that chromosome if the hybridization capacity of the shared repetitive sequences is reduced by incubation with unlabeled human genomic DNA. In Section VI.A., the nucleic acid sequences of the heterogeneous

mixture were cloned in the phage vector Charon 21A, in which the ratio of insert of vector DNA is about 0.1 (4 kb average insert to 40 kb of vector). In this section, we demonstrate that transferring the same inserts to a smaller cloning vector, the about 3 kb Bluescribe plasmid, which increases the ratio of insert to vector DNA to 0.5, improved the specificity and intensity of the staining.

As previously discussed, incubation of the probe can be carried out with the probe alone, with the probe mixed with unlabeled genomic DNA, and with the probe mixed with unlabeled DNA enriched in all or some shared repetitive sequences. If unlabeled genomic DNA is added, then it is important to add enough to incapacitate sufficiently the shared repetitive sequences in the probe. However, the genomic DNA also contains unlabeled copies of the sequences, the hybridization of which is desired. As explained above, Q is herein defined as the ratio of unlabeled to labeled copies of the chromosome-specific sequences in the hybridization mixture.

Pinkel et al., PNAS (USA), 85: 9138-9142 (December 1988) describes the use of unlabeled genomic DNA to competitively inhibit hybridization of those sequences in a chromosome-specific library that are shared with other chromosomes. That paper describes materials and methods for fluorescence *in situ* hybridization with human chromosome-specific libraries [chromosome 4 library LL04NS02 subcloned into Bluescribe plasmids (pBS-4); chromosome 21 library LL21NS02 subcloned into Bluescribe plasmids (pBS-21)]. The results of those hybridizations are shown in Figure 4 A-C and 4F and G.

VI.C. Hybridization of Yeast Artificial Chromosomes (YACS) to Human Metaphase Spread

YACS. Seven yeast clones HY1, HY19, HY29, HYA1.A2, HYA3.A2, HYA3.A9, and HYA9.E6 were obtained from D. Burke (Washington University, St. Louis, MO). The lengths of the human DNA in the clones ranged from about 100 kb to about 600 kb. Gel electrophoresis was performed to verify the size of these inserts. Each of these clones was grown up and total DNA was isolated. The isolated DNA was biotinylated by nick translation so that 10-30% of the thymidine was replaced by biotin-11-dUTP. The concentration of the total labeled DNA after nick translations is in the range of 10-20 ng/ μ l.

Blocking DNA. Human placental DNA (Sigma) was treated with proteinase K and extracted with phenol and sonicated to a size range of 200-600 bp. Total DNA isolated from yeast not containing an artificial chromosome was sonicated to a similar size range. Both of these DNA's were maintained at a concentration of 1-10 μ g/ μ l.

Fluorescence *in situ* hybridization (FISH). Hybridization followed the procedures of Pinkel et al. (1988), *supra* with slight modifications. Metaphase spreads were prepared from methotrexate synchronized cultures according to the procedures of Harper et al. PNAS (USA) 78: 4458-4460, (1981). Cells were fixed in methanol/acetic acid, fixed (3: 1), dropped onto slides, air dried, and stored at -20°C under nitrogen gas until used. The slides were then immersed two minutes in 70% formamide/2xSSC to denature the target DNA sequences, dehydrated in a 70-85-100% ethanol series, and air dried. (SSC is 0.15 M NaCl/0.015 M'Na Citrate, pH 7). Ten - 100 ng of biotinylated yeast DNA, and approximately 1 μ g each of unlabeled yeast and human genomic DNA were then added to the hybridization mix (final volume 10 μ l, final composition 50% formamide/2xSSC/10% dextran sulfate), heated to 70°C for 5 min., and then incubated at 37°C for 1 hr to allow the complementary strands of the more highly repeated sequences to reassociate.

The hybridization mixture was then applied to the slide (approximately 4 cm^2 area) and sealed with rubber cement under a glass cover slip. After overnight incubation at 37°C the coverslip was removed and the slide washed 3 times 3 min each in 50% formamide/2xSSC at 42-45°C, and once in PN buffer [mixture of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 to give pH 8; 0.1% Nonidet P-40 (Sigma)]. The bound probe was then detected with alternating 20 min incubations (room temperature in avidin-FITC and goat anti-avidin antibody, both at 5 μ g/ml in PNM buffer (PN buffer plus 5% nonfat dry milk, centrifuged to remove solids; 0.02% Na azide). Avidin and anti-avidin incubation were separated by 3 washes of 3 min each in PN buffer. Two or three layers of avidin were applied (Avidin, DCS grade, and biotinylated goat anti-avidin are obtained from Vector Laboratories Inc., Burlingame, CA).

Figure 5 shows the hybridization of HYA3.A2 (580 kb of human DNA) to 12q21.1. The location of the hybridization was established by using a conventional fluorescent banding technique employing the DAPI/actinomycin D procedure: Schweizer, "Reverse fluorescent chromosome banding with chromomycin and DAPI," Chromosoma, 58: 307-324 (1976). The hybridization signal forms a band across the width of each of the chromosome 12s, indicating the morphology of the packing of DNA in that region of the chromosome.

The YAC clone positions are attributed as shown in Table 2 below.

Table 2

| YAC Competition Hybridization | | |
|-------------------------------|-------------|--------------|
| YAC Clone | Insert Size | Localization |
| HY1 | 120 | Xq23 |

Table 2 (continued)

| YAC Competition Hybridization | | |
|-------------------------------|-------------|-------------------|
| YAC Clone | Insert Size | Localization |
| Hy19 | 450 | 8q23.3 21q21.1 |
| HY29 | 500 | 14q12 |
| HYA1.A2 | 250 | 6q16 |
| HYA3.A2 | 580 | 12q21.1 |
| HYA3.A9 | 600 | 14q21 |
| HYA9.E6 | 280 | 1p36.2 3q22 |

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VI.D. Hybridization With Human/Hamster Hybrid Cell

Essentially the same hybridization and staining conditions were used in this example as for those detailed in the procedure of Pinkel et al. (1988), *supra* and exemplified in Sections V.C. and VI.B., *supra*. In this example, 400 ng of biotin labeled DNA from a hamster-human hybrid cell that contains one copy of human chromosome 19 was mixed with 1.9 ug of unlabeled human genomic DNA in 10 ul of hybridization mix. Hybridization was for approximately 60 hours at 37°C. Fluorescent staining of the bound probe and counterstaining of the chromosomes was as in the other examples above. Figure 6 shows the results of the hybridization.

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VII. Specific Applications.

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The present invention allows microscopic and in some cases flow cytometric detection of genetic abnormalities on a cell by cell basis. The microscopy can be performed entirely by human observers, or include various degrees of additional instrumentation and computational assistance, up to full automation. The use of instrumentation and automation for such analyses offers many advantages. Among them are the use of fluorescent dyes that are invisible to human observers (for example, infrared dyes), and the opportunity to interpret results obtained with multiple labeling methods which might not be simultaneously visible (for example, combinations of fluorescent and absorbing stains, autoradiography, etc.) Quantitative measurements can be used to detect differences in staining that are not detectable by human observers. As is described below, automated analysis can also increase the speed with which cells and chromosomes can be analysed.

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In accordance with the present invention, translocations can be detected in interphase nuclei by the abnormal juxtaposition of hybridization domains that are normally separate following hybridization with probes that flank or span the region(s) of the chromosome(s) that are at the point(s) of rearrangement. Translocations involve at least two different chromosome types and result in derivative chromosomes possessing only one centromere each. Dicentrics involve at least two different chromosome types and result in at least one chromosome fragment lacking a centromere and one having two centromeres. Inversions involve a reversal of polarity of a portion of a chromosome.

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VII.A Banding Analysis

Substantial effort has been devoted during the past thirty years to development of automated systems (especially computer controlled microscopes) for automatic chromosome classification and aberration detection by analysis of metaphase spreads. In recent years, effort has been directed at automatic classification of chromosomes which have been chemically stained to produce distinct banding patterns on the various chromosome types. These efforts have only partly succeeded because of the subtle differences in banding pattern between chromosome types of approximately the same size, and because differential contraction of chromosomes in different metaphase spreads causes a change in the number and width of the bands visible on chromosomes of each type. The present invention overcomes these problems by allowing construction of reagents which produce a staining pattern whose spacing, widths and labeling differences (for example different colors) are optimized to facilitate automated chromosome classification and aberration detection. This is possible because hybridization probes can be selected as desired along the lengths of the chromosomes. The size of a band produced by such a reagent may range from a single small dot to a substantially uniform coverage of one or more whole chromosomes. Thus the present invention allows construction of a hybridization probe and use of labeling means, preferably fluorescence, such that adjacent hybridization domains can be distinguished, for example by color, so that bands too closely spaced to be resolved spatially can be detected spectrally (i.

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e. if red and green fluorescing bands coalesce, the presence of the two bands can be detected by the resulting yellow fluorescence).

The present invention also allows construction of banding patterns tailored to particular applications. Thus they can be significantly different in spacing and color mixture, for example, on chromosomes that are similar in general shape and size and which have similar banding patterns when conventional techniques are used. The size, shape and labeling (e.g. color) of the hybridization bands produced by the probes of the present invention can be optimized to eliminate errors in machine scoring so that accurate automated aberration detection becomes possible. This optimized banding pattern will also greatly improve visual chromosome classification and aberration detection.

The ease of recognition of specific translocation breakpoints can be improved by using a reagent closely targeted to the region of the break. For example, a high complexity probe of this invention comprising sequences that hybridize to both sides of the break on a chromosome can be used. The portion of the probe that binds to one side of the break can be detected differently than that which binds to the other, for example with different colors. In such a pattern, a normal chromosome would have the different colored hybridization regions next to each other, and such bands would appear close together. A break would separate the probes to different chromosomes or result in chromosomal fragments, and could be visualized as much further apart on an average.

VII.B Biological Dosimetry

One approach to biological dosimetry is to measure frequencies of structurally aberrant chromosomes as an indication of the genetic damage suffered by individuals exposed to potentially toxic agents. Numerous studies have indicated the increase in structural aberration frequencies with increasing exposure to ionizing radiation and other agents, which are called clastogens. Dicentric chromosomes are most commonly scored because their distinctive nature allows them to be scored rapidly without banding analysis. Rapid analysis is important because of the low frequency of such aberrations in individuals exposed at levels found in workplaces ($\sim 2 \times 10^{-3}/\text{cell}$). Unfortunately, dicentrics are not stably retained so the measured dicentric frequency decreases with time after exposure. Thus low level exposure over long periods of time does not result in an elevated dicentric frequency because of the continued clearance of these aberrations. Translocations are better aberrations to score for such dosimetric studies because they are retained more or less indefinitely. Thus, assessment of genetic damage can be made at times long after exposure. Translocations are not routinely scored for biological dosimetry because the difficulty of recognizing them makes scoring sufficient cells for dosimetry logically impossible.

The present invention eliminates this difficulty. Specifically, hybridization with a probe which substantially uniformly stains several chromosomes (e.g. chromosomes 1, 2, 3 and 4) allows immediate microscopic identification in metaphase spreads of structural aberrations involving these chromosomes. Normal chromosomes appear completely stained or unstained by the probe. Derivative chromosomes resulting from translocations between targeted and non-targeted chromosomes are recognized as being only partly stained, Fig. 4D. Such partially hybridized chromosomes can be immediately recognized either visually in the microscope or in an automated manner using computer assisted microscopy. Discrimination between translocations and dicentrics is facilitated by adding to the probe, sequences found at all of the chromosome centromeres. Detection of the centromeric components of the probe with a labeling means, for example color, different from that used to detect the rest of the probe elements allows ready identification of the chromosome centromeres, which in turn facilitates discrimination between dicentrics and translocations. This technology dramatically reduces the scoring effort required with previous techniques so that it becomes feasible to examine tens of thousands of metaphase spreads as required for low level biological dosimetry.

VII.C. Prenatal Diagnosis

The most common aberrations found prenatally are trisomies involving chromosomes 21 (Down syndrome), 18 (Edward syndrome) and 13 (Patau syndrome) and X0 (Turner syndrome), XXY (Klinefelter syndrome) and XYY disease. Structural aberrations also occur. However, they are rare and their clinical significance is often uncertain. Thus, the importance of detecting these aberrations is questionable. Current techniques for obtaining fetal cells for conventional karyotyping, such as, amniocentesis and chorionic villus biopsy yield hundreds to thousands of cells for analysis. These are usually grown in culture for 2 to 5 weeks to produce sufficient mitotic cells for cytogenetic analysis. Once metaphase spreads are prepared, they are analyzed by conventional banding analysis. Such a process can only be carried out by highly skilled analysts and is time consuming so that the number of analyses that can be reliably carried out by even the largest cytogenetics laboratories is only a few thousand per year. As a result, prenatal cytogenetic analysis is usually limited to women whose children are at high risk for genetic disease (e.g. to women over the age of 35).

The present invention overcomes these difficulties by allowing simple, rapid identification of common numerical chromosome aberrations in interphase cells with no or minimal cell culture. Specifically, abnormal numbers of chromosomes 21, 18, 13, X and Y can be detected in interphase nuclei by counting numbers of hybridization domains

following hybridization with probes specific for these chromosomes (or for important regions thereof such as 21q22 for Down syndrome). A hybridization domain is a compact, distinct region over which the intensity of hybridization is high. An increased frequency of cells showing three domains (specifically to greater than 10%) for chromosomes 21, 18 and 13 indicates the occurrence of Down, Edward and Patau syndromes, respectively. An increase in the number of cells showing a single X-specific domain and no Y-specific domain following hybridization with X-specific and Y-specific probes indicates the occurrence of Turner syndrome. An increase in the frequency showing two X-specific domains and one Y-specific domain indicates Kleinfelter syndrome, and increase in the frequency of cells showing one X-specific domain and two Y-specific domains indicates an XYY fetus. Domain counting in interphase nuclei can be supplemented (or in some cases replaced) by measurement of the intensity of hybridization using, for example, quantitative fluorescence microscopy or flow cytometry, since the intensity of hybridization is approximately proportional to the number of target chromosomes for which the probe is specific. Numerical aberrations involving several chromosomes can be scored simultaneously by detecting the hybridization of the different chromosomes with different labeling means, for example, different colors. These aberration detection procedures overcome the need for extensive cell culture required by procedures since all cells in the population can be scored. They eliminate the need for highly skilled analysts because of the simple, distinct nature of the hybridization signatures of numerical aberrations. Further, they are well suited to automated aberration analysis.

The fact that numerical aberrations can be detected in interphase nuclei also allows cytogenetic analysis of cells that normally cannot be stimulated into mitosis. Specifically, they allow analysis of fetal cells found in maternal peripheral blood. Such a feature is advantageous because it eliminates the need for invasive fetal cell sampling such as amniocentesis or chorionic villus biopsy.

As indicated in the Background, the reason such embryo-invasive methods are necessary is that conventional karyotyping and banding analysis requires metaphase chromosomes. At this time, there are no accepted procedures for culturing fetal cells separated from maternal blood to provide a population of cells having metaphase chromosomes. In that the staining reagents of this invention can be employed with interphase nuclei, a non-embryo-invasive method of karyotyping fetal chromosomes is provided by this invention.

The first step in such a method is to separate fetal cells that have passed through the placenta or that have been shed by the placenta into the maternal blood. The incidence of fetal cells in the maternal bloodstream is very low, on the order of 10^{-4} to 10^{-6} cells/ml and quite variable depending on the time of gestation; however, appropriately marked fetal cells may be distinguished from maternal cells and concentrated, for example, with high speed cell sorting.

The presence of cells of a male fetus may be identified by a label, for example a fluorescent tag, on a chromosome-specific staining reagent for the Y chromosome. Cells that were apparently either lymphocytes or erythrocyte precursors that were separated from maternal blood were shown to be Y-chromatin-positive. [Zillacus et al., Scan. J. Haematol, 15: 333 (1975); Parks and Herzenberg, Methods in Cell Biology, Vol. 10, pp. 277-295 (Academic Press, N.Y., 1982); and Siebers et al., Humangenetik, 28: 273 (1975)].

A preferred method of separating fetal cells from maternal blood is the use of monoclonal antibodies which preferentially have affinity for some component not present upon the maternal blood cells. Fetal cells may be detected by paternal HLA (human leukocyte antigen) markers or by an antigen on the surface of fetal cells. Preferred immunochemical procedures to distinguish between fetal and maternal leukocytes on the basis of differing HLA type use differences at the HLA-A2, -A3, and -B7 loci, and further preferred at the -A2 locus. Further, first and second trimester fetal trophoblasts may be marked with antibody against the internal cellular constituent cytokeratin which is not present in maternal leukocytes. Exemplary monoclonal antibodies are described in the following references.

Herzenberg et al., PNAS, 76: 1453 (1979), reports the isolation of fetal cells, apparently of lymphoid origin, from maternal blood by fluorescence activated cell sorting (FACS) wherein the separation was based on the detection of labeled antibody probes which bind HLA-A2 negative cells in maternal blood. Male fetal cells separated in that manner were further identified by quinacrine staining of Y-chromatin.

Covone et al., Lancet, Oct. 13, 1984: 841, reported the recovery of fetal trophoblasts from maternal blood by flow cytometry using a monoclonal antibody termed H315. Said monoclonal reportedly identifies a glycoprotein expressed on the surface of the human syncytiotrophoblast as well as other trophoblast cell populations, and that is absent from peripheral blood cells.

Kawata et al., J. Exp. Med., 160: 653 (1984), discloses a method for isolating placental cell populations from suspensions of human placenta. The method uses coordinate two-color and light-scatter FACS analysis and sorting. Five-different cell populations were isolated on the basis of size and quantitative differences in the coordinate expression of cell surface antigens detected by monoclonal antibodies against an HLA-A, B, C monomorphic determinant (MB40.5) and against human trophoblasts (anti-Trop-1 and anti-Trop-2).

Loke and Butterworth, J. Cell Sci., 76: 189 (1985), describe two monoclonal antibodies, 18B/A5 and 18A/C4, which are reactive with first trimester cytotrophoblasts and other fetal epithelial tissues including syncytiotrophoblasts.

A preferred monoclonal antibody to separate fetal cells from maternal blood for staining according to this invention is the anti-cytokeratin antibody Cam 5.2, which is commercially available from Becton-Dickinson (Franklin Lakes, N.

J., USA).

Other preferred monoclonal antibodies for separating fetal cells from maternal blood are those disclosed in co-pending, commonly owned US Patent Application, USSN 389,224, filed August 3, 1989, entitled "Method for Isolating Fetal Cytotrophoblast Cells". [See also: in Fisher et al., *J. Cell. Biol.*, 109 (2): 891-902 (1989)]. The monoclonal antibodies disclosed therein react specifically with antigen on first trimester human cytotrophoblast cells, which fetal cells have the highest probability of reaching the maternal circulation. Said application and article are herein specifically incorporated by reference. Briefly, the disclosed monoclonal antibodies were raised by injection of test animals with cytotrophoblast cells obtained from sections of the placental bed, that had been isolated by uterine aspiration. Antibodies raised were subjected to several cytological screens to select for those antibodies which react with the cytotrophoblast stem cell layer of first trimester chorionic villi.

Preferred monoclonal antibodies against such first trimester cytotrophoblast cells disclosed by Fisher et al. include monoclonal antibodies produced from the following hybridomas deposited at the American Type Culture Collection (ATCC; Rockville, MD, USA) under the Budapest Treaty:

| Hybridoma | ATCC Accession # |
|-----------|------------------|
| J1D8 | HB10096 |
| P1B5 | HB10097 |

Both hybridoma cultures were received by the ATCC on April 4, 1989 and reported viable thereby on April 14, 1989.

Fisher et al. state that fetal cells isolated from maternal blood by use of said monoclonal antibodies are capable of replication in vitro. Therefore, fetal cells isolated by the method of Fisher et al., that is, first trimester fetal cytotrophoblasts, may provide fetal chromosomal material that is both in metaphase and in interphase.

The fetal cells, preferably leukocytes and cytotrophoblasts, more preferably cytotrophoblasts, once marked with an appropriate antibody are then separated from the maternal cells either directly or by preferably separating and concentrating said fetal cells by cell sorting or panning. For example, FACS may be used to separate fluorescently labeled fetal cells, or flow cytometry may be used.

The fetal cells once separated from the maternal blood can then be stained according to the methods of this invention with appropriate chromosome-specific staining reagents of this invention, preferably those of particular importance for prenatal diagnosis. Preferred staining reagents are those designed to detect aneuploidy, for example, trisomy of any of several chromosomes, including chromosome types 21, 18, 13, X and Y and subregions on such chromosomes, such as, subregion 21q22 on chromosome 21.

Preferably, a fetal sample for staining analysis according to this invention comprises at least 10 cells or nuclei, and more preferably about 100 cells or nuclei.

35 VII.D Tumor Cytogenetics

Numerous studies in recent years have revealed the existence of structural and numerical chromosome aberrations that are diagnostic for particular disease phenotypes and that provide clues to the genetic nature of the disease itself. Prominent examples include the close association between chronic myelogenous leukemia and a translocation involving chromosome 9 and 22, and the association of a translocation involving chromosomes 8 and 14 with Burkitt's lymphoma. Current progress in elucidating new tumor specific abnormalities is limited by the difficulty of producing representative, high quality banded metaphase spreads for cytogenetic analysis. These problems stem from the fact that many human tumors are difficult or impossible to grow in culture. Thus, obtaining mitotic cells is usually difficult. Even if the cells can be grown in culture, there is the significant risk that the cells that do grow may not be representative of the tumorigenic population. That difficulty also impedes the application of existing genetic knowledge to clinical diagnosis and prognosis.

The present invention overcomes these limitations by allowing detection of specific structural and numerical aberrations in interphase nuclei. These aberrations are detected as described supra. Hybridization with whole chromosome probes will facilitate identification of previously unknown aberrations thereby allowing rapid development of new associations between aberrations and disease phenotypes. As the genetic nature of specific malignancies becomes increasingly well known, the interphase assays can be made increasingly specific by selecting hybridization probes targeted to the genetic lesion. Translocations at specific sites on selected chromosomes can be detected by using hybridization probes that closely flank the breakpoints. Use of these probes allows diagnosis of these specific disease phenotypes. Translocations may be detected in interphase because they bring together hybridization domains that are normally separated, or because they separate a hybridization domain into two, well separated domains. In addition, they may be used to follow the reduction and reemergence of the malignant cells during the course of therapy. Interphase analysis is particularly important in such an application because of the small number of cells that may be present

and because they may be difficult or impossible to stimulate into mitosis.

Duplications and deletions, processes involved in gene amplification and loss of heterozygosity, can also be detected in metaphase spreads and interphase nuclei using the techniques of this invention. Such processes are implicated in an increasing number of different tumors.

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VIII. Detection of BCR-ABL Fusion in Chronic Myelogenous Leukemia (CML)

Probes. This section details a CML assay based upon FISH with probes from chromosomes 9 and 22 that flank the fused BCR and ABL sequences in essentially all cases of CML (Figure 8). The BCR and ABL probes used in the examples of this section were kindly provided by Carol A. Westbrook of the Department of Medicine, Section of Hematology/Oncology at the University of Chicago Medical Center in Chicago, Illinois (USA).

The ABL probe on chromosome 9, c-hu-ABL, is a 35-kb cosmid (pCV105) clone selected to be telomeric to the 200-kb region of ABL between exons IB and II in which the breaks occur (24). The BCR probe on chromosome 22, PEM12, is an 18-kb phage clone (in EMBL3) that contains part of, and extends centromeric to, the 5.8-kb breakpoint cluster region of the BCR gene in which almost all CML breakpoints occur. FISH was carried out using a biotin labeled ABL probe, detected with the fluorochrome Texas red, and a digoxigenin labeled BCR probe, detected with the green fluorochrome FITC. Hybridization of both probes could be observed simultaneously using a fluorescence microscope equipped with a double band pass filter set (Omega Optical).

Figure 8 is a schematic representation of the BCR gene on chromosome 22, the ABL gene of chromosome 9, and the BCR-ABL fusion gene on the Philadelphia chromosome, showing the location of CML breakpoints and their relation to the probes. Exons of the BCR gene are depicted as solid boxes. The Roman numeral I refers to the first exon of the BCR gene; the arabic numerals 1-5 refer to the exons within the breakpoint cluster region, here indicated by the dashed line. The approximate location of the 18 kb phage PEM12 probe (the BCR probe) is indicated by the open horizontal bar. Since the majority of breakpoints in CML occur between exons 2 and 4, 15 kb or more of target for PEM12 will remain on the Philadelphia chromosome. In the classic reciprocal translocation a few kb of target for PEM12 (undetectable fluorescent signal) will be found on the derivative chromosome. The map and exon numbering (not to scale) is adapted from Heisterkamp et al. (ref. 34, supra).

Exons of the ABL gene are depicted as open vertical bars (not to scale). The Roman numerals Ia and Ib refer to the alternative first exons, and II to the second exon. Exon II is approximately 25 kb upstream of the end of the 28 kb cosmid c-hu-abl (the ABL probe). All CML breakpoints occur upstream of exon II, usually between exons Ib and Ia, within a region that is approximately 200 kb in length. Thus, c-hu-abl will always be 25 to 200 kb away from the fusion junction. The map (not to scale) is adapted from Heisterkamp et al. (ref. 35, supra). The BCR-ABL fusion gene is depicted. In CML, PEM12 will always lie at the junction, and c-hu-abl will be separated from PEM12 by 25 to 225 kb.

Sample Preparation: CML-4: Peripheral blood was centrifuged for 5 min. Ten drops of interface was diluted with PBS, spun down, fixed in methanol/acetic acid (3: 1), and dropped on slides. CML-2, 3, 7: Five to 10 drops of marrow diluted with PBS to prevent clotting were fixed in methanol/acetic acid and dropped on slides. CML-1, 4, 5, 6: Peripheral blood and/or bone marrow was cultured in RPMI 1640 supplemented with 10% fetal calf serum, an antibiotic mixture (gentamycin 500 mg/ml), and 1% L-glutamine for 24h. Cultures were synchronized according to J.J. Yunis and M.E. Chandler *Prog. in Clin. Path.*, 7: 267 (1977), and chromosome preparations followed Gibis and Jackson, *Karyogram*, 11: 91 (1985).

Hybridization and Detection Protocol. Hybridization followed procedures described by D. Pinkel et al. (27), Trask et al. (25), and J. B. Lawrence et al (30), with modifications. The BCR probe was nick-translated (Bethesda Research Laboratories Nick-Translation System) with digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals) with an average incorporation of 25%. The ABL probe was similarly nick-translated with biotin-11-dUTP (Enzo Diagnostics).

1. **Hybridization.** Denature target interphase cells and/or metaphase spreads on glass slides at 72°C in 70% formamide/2xSSC at pH 7 for 2 min. Dehydrate in an ethanol series (70%, 85%, and 100% each for 2 min.). Air dry and place at 37°C (2xSSC is 0.3M NaCl/30 mM sodium citrate). Heat 10 ml of hybridization mixture containing 2 ng/ml of each probe, 50% formamide/2xSSC, 10% dextran sulphate, and 1 mg/ml human genomic DNA (sonicated to 200-600 bp) to 70°C for 5 min. to denature the DNA. Incubate for 30 min. at 37°C. Place on the warmed slides, cover with a 20 mm x 20 mm coverslip, seal with rubber cement, and incubate overnight in a moist chamber at 37°C. Remove coverslips and wash three times for 20 minutes each in 50% formamide/2xSSC pH 7 at 42°C, twice for 20 minutes each in 2xSSC at 42°C, and finally rinse at room temperature-in 4xSSC.

2. **Detection of Bound Probes:** All incubation steps are performed with approximately 100 ml of solution at room temperature under coverslips. The biotinylated ABL probe was detected first, then the digoxigenin-labeled BCR probe.

a. **Biotinylated ABL Probe:** Preblock with 4xSSC/1% bovine serum albumin (BSA) for 5 min. Apply Texas Red-avidin (Vector Laboratories Inc., 2 mg/ml in 4xSSC/1% BSA) for 45 min. Wash in 4xSSC once, 4xSSC/1% Triton-X 100 (Sigma) and then again in 4xSSC, 5 min. each. Preblock for 5 min. in PNM (PN containing 5% non-fat dry milk and 0.02% sodium azide and centrifuged to remove solids. PN is 0.1 M NaH₂PO₄/0.1M Na₂HPO₄, 0.05% NP40, pH

8). Apply biotinylated goat anti-avidin (Vector Laboratories Inc., 5 mg/ml in PNM) for 45 min. Wash twice in PN for 5 min. Apply a second layer of Texas Red-avidin (2 mg/ml in PNM) for 45 min. Wash twice in PN for 5 min. each.

b. Digoxigenin-Labeled BCR Probe: Preblock with PNM for 5 min. Apply sheep anti-digoxigenin antibody (obtained from D. Pepper, Boehringer Mannheim Biochemicals, Indianapolis, IN; 15.4 mg/ml in PNM) for 45 min. Wash twice in PN for 5 min. each. Preblock with PNM for 5 min. Apply rabbit-anti-sheep antibody conjugated with FITC (Organon Teknika- Cappel, 1: 50 in PNM) for 45 min. Wash twice for 5 min. each in PN. If necessary, the signal is amplified by preblocking for 5 min. with PNM and applying sheep anti-rabbit IgG antibody conjugated to FITC (Organon Teknika- Cappel, 1: 50 in PNM) for 45 min. Rinse in PN.

10 3. Visualization: The slides are mounted fluorescence antifade solution [G. D. Johnson and J. G. Nogueria, *J. Immunol. Methods*, 43: 349 (1981)] (ref. 31, *supra*) containing 1 mg/ml 4',6-amidino-2-phenylindole (DAPI) as a counterstain, and examined using a FITC/Texas red double-band pass filter set (Omega Optical) on a Zeiss Axioskop.

The method used for BCR-ABL PCR tested herein was that described in Hegewisch-Becker et al. for CML-3, 4 and 7 (ref. 32, *supra*), and Kohler et al., for CML-5 and 6 (ref. 33, *supra*).

15 Results. ABL and BCR hybridization sites were visible on both chromatids of chromosomes in most metaphase spreads. The ABL probe bound to metaphase spreads from normal individuals (Figure 9 A) near the telomere on 9q while the BCR probe bound at 22q11 (Figure 9B). Hybridization with the ABL or BCR probe to normal interphase nuclei typically resulted in two tiny fluorescent dots corresponding to the target sequence on both chromosome homologues. The spots were apparently randomly distributed in the two dimensional nuclear images and were usually well separated. A few cells showed two doublet hybridization signals probably a result of hybridization to both sister chromatids of both 20 homologues in cells which had replicated this region of DNA (i.e., those in the S- or G2- phase of cell cycle). Dual color FISH of the ABL (red) and BCR (green) probes to normal G1 nuclei yielded two red (ABL) and two green (BCR) hybridization signals distributed randomly around the nucleus.

25 The genetic rearrangement of CML brings the DNA sequences homologous to the probes together on an abnormal chromosome, usually the Ph¹, and together in the interphase nucleus, as illustrated in Figure 8. The genomic distance between the probe binding sites in the fusion gene varies among CML cases, ranging from 25 to 225 kb, but remains the same in all the cells of a single leukemic clone. Dual color hybridization with ABL and BCR probes to interphase CML cells resulted in one red and one green hybridization signal located at random in the nucleus, and one red-green doublet signal in which the separation between the two colors was less than 1 micron (or one yellow hybridization signal for hybridization in very close proximity, see Figure 10). The randomly located red and green signals are ascribed 30 to hybridization to the ABL and BCR genes on the normal chromosomes, and the red-green doublet signal to hybridization to the BCR-ABL fusion gene. Interphase mapping studies suggest that DNA sequences separated by less than 250 kb should be separated in interphase nuclei by less than 1 micron (25). As a result, cells showing red and green hybridization signals separated by greater than 1 micron were scored as normal since this is consistent with the hybridization sites being on different chromosomes. However, due to statistical considerations, some normal cells will have red and green dots close enough together to be scored as abnormal. In these two dimensional nuclear analyses, 35 9 out of 750 normal nuclei had red and green hybridization signals less than 1 micron of each other. Thus, approximately 1% of normal cells were classified as abnormal.

40 Table 3 shows the hybridization results for 7 samples from 6 CML cases along with conventional karyotypes, and other diagnostic results (PCR and Southern blot data). All six cases, including 3 that were found to be Ph¹ negative by banding analysis (CML-5, -6 and -7), showed red-green hybridization signals separated by less than 1 micron in greater than 50% of nuclei examined. In most, the fusion event was visible in almost every cell. One case (CML-7) showed fusion signals in almost every cell even though PCR analysis failed to detect the presence of a fusion gene and banding analysis did not reveal a Philadelphia chromosome.

Table 3

| Sample | Cytogenetics | Fluorescence in situ hybridization Metaphase | Fluorescence in situ hybridization Interphase nuclei | Other information |
|----------------------|---------------------------|--|--|---|
| | | | | |
| CML-1 ^a | 46XX, t(9;22)(q34;q11) | Hybridization to telomere of small acrocentric | 80% showed red-green fusion 2% showed red-green doublets 18% not interpretable | |
| CML-2 ^d | 46XY, t(9;22)(q34;q11) | Not available | 60% showed red-green fusion Hybridization efficiency was low | |
| CML-3 ^{d,e} | 46XY, t(9;22)(q34;q11) | Not available | 75% showed red-green fusion 25% appeared normal | BCR-ABL fusion positive by PCR |
| CML-4 ^{d,e} | 46XY, t(9;22)(q34;q11) | Not available | 100% showed red-green fusion | BCR-ABL fusion positive by PCR |
| CML-5 ^c | 47XY,+8,del(22)(q11) | 47 chromosomes. Red-green fusion at telomere of small acrocentric | 100% showed red-green fusion | BCR-ABL fusion positive by PCR |
| CML-6 ^a | 46XY ins(22;9)q11;q34,q2? | Red-green fusion interstitial on small acrocentric | 100% showed red-green fusion | BCR-ABL fusion positive by PCR |
| CML-7 ^b | 46XY t(5;9)q(?;q?) | Not available | 100% showed red-green fusion | BCR-ABL fusion negative in two tests by PCR BCR rearrangement detected by Southern blot analysis |

Clinical data: ^a CML, chronic phase receiving no treatment; ^b CML, chronic phase receiving hydroxyurea; ^c CML, blast crisis receiving no treatment; ^d CML, blast crisis receiving hydroxyurea; and ^e CML-3 and CML-4 represent respectively bone marrow and blood samples from one patient.

Hybridization to metaphase spreads was performed in three cases (CML-1,-5 and -6). All of these showed red and

green hybridization signals in close proximity on a single acrocentric chromosome. In two cases, scored as t(9; 22) (q34; q11) by banding, the red-green pair was in close proximity to the telomere of the long arm of a small acrocentric chromosome as expected for the Ph¹ (Figure 9C). One case (CML-6) was suspected by trassical cytogenetics to have an insertion of chromosomal material at 22q11. Dual color hybridization to metaphase spreads from this case showed the red-green pair to be centrally located in a small chromosome (Figure 9D). That result is consistent with formation of the BCR-ABL fusion gene by an insertion. In one case (CML-1), two pairs of red-green doublet signals were seen in 3 out 150 (2%) interphase nuclei. That may indicate a double Ph¹ (or double fusion gene) in those cells. Such an event was not detected by standard cytogenetics, which was limited to analysis of 25 metaphase spreads. The acquisition of an additional Ph¹ is the most frequent cytogenetic event accompanying blast transformation, and its cytogenetic detection may herald disease acceleration.

Simultaneous hybridization with ABL and BCR probes to metaphase spreads of the CML derived cell line K-562 showed multiple red-green hybridization sites along both arms of a single acrocentric chromosome. Hybridization to interphase nuclei showed that the red and green signals were confined to the same region of the nucleus. That is consistent with their being localized on a single chromosome. Twelve to fifteen hybridization pairs were seen in each nucleus indicating corresponding amplification of the BCR-ABL fusion gene (see Figures 9E and 9F). These findings are consistent with previous Southern blot data showing amplification of the fusion gene in this cell line (26).

In summary, analysis of interphase cells for seven CML, and four normal cell samples using dual color FISH with ABL and BCR probes suggests the utility of this approach for routine diagnosis of CML and clinical monitoring of the disease. Among its very important advantages are the ability to obtain genetic information from individual interphase or metaphase cells in less than 24 hours. Thus, it can be applied to all cells of a population, not just to those that fortuitously or through culture, happen to be in metaphase. Further, the genotypic analysis can be associated with cell phenotype, as judged by morphology or other markers, thereby permitting the study of lineage specificity of cells carrying the CML genotype as well as assessment of the frequency of cells carrying the abnormality.

Random juxtaposition of red and green signals in two dimensional images of normal cells, which occurs in about 0.01 of normal cells, sets the low frequency detection limit. That detection limit may be lowered by more complete quantitative measurement of the separation and intensity of the hybridization signals in each nucleus using computerized image analysis. Such analysis will be particularly important in studying patient populations in which the cells carrying the BCR-ABL fusion at low frequency (e.g., during remission, after bone marrow transplantation, during relapse or in model systems).

This assay also should be advantageous for detection of CML cells during therapy when the number of cells available for analysis is low since only a few cells are required. Finally, simple counting of hybridization spots allows for the detection and quantitative analysis of amplification of the BCR-ABL fusion gene as illustrated for the K562 cell line (Figure 9E). Quantitative measurement of fluorescence intensity may assist with such an analysis.

IX. Method of Preparing and Applying Single Stranded Nucleic Acid Probes to Double Standed Target DNAs

Generally the method for preparing and applying single stranded DNA hybridization probes to double stranded target DNA involves treating both target DNA and probe DNA with the same restriction endonuclease followed by digestion of single strands adjacent to the restriction cuts. Probes are constructed by resynthesizing the digested single strands with labeled nucleotides. The labeled strands are substantially complementary to the undigested single strands of the target DNAs. The double stranded DNA fragments containing the labeled single strands are broken into smaller pieces and denatured. The hybridization probes are obtained by separating the labeled single stranded fragments from the unlabeled fragments.

DNA to be used in the probes is treated with a restriction endonuclease to form restriction fragments having "sticky" ends. That is, it is important that the restriction endonuclease make a staggered cut through the double stranded DNA. Suitable restriction endonuclease include, but are not limited to, Hind III, Bam H1, Eco R1, or the like, all of which are commercially available, e.g., Promega Biotec (Madison, WI), or Boehringer Mannheim (Indianapolis, IN). In selecting a restriction endonuclease it is preferable that the resulting restriction fragments be within a size range which allows them to be directly inserted into available cloning vectors. Suitable cloning vectors included plasmids, such as pBR322, and phages, such as lambda phage, various derivatives of both of these being commercially available, e.g., Promega Biotec (Madison, WI), and Boehringer Mannheim (Indianapolis, IN). Amplified copies of the restriction fragments are isolated using standard techniques, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring-Harbor Laboratory, 1982). Alternatively, for some applications restriction fragments can be obtained from existing libraries. For example, the American Type Culture Collection, Rockville, MD, holds collections of human chromosome-specific libraries of restriction fragments which are available to the public.

Standard procedures are followed in treating the restriction fragments with exonuclease, and in enzymatically resynthesizing the digested strands in the presence of labeled precursors. In particular the technique disclosed by James and Leffak, Anal. Biochem., Vol. 141, pp. 33-37 (1984), is followed. Briefly, to the restriction fragments about 3 units

of exonuclease III are added per microgram of DNA in a solution consisting of 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂ and 1 mM dithiothreitol, at 37°C. Digestion is terminated by heating the sample to 60°C for 5-10 minutes. James and Laffak report that these conditions result in the digestion of about 80-200 nucleotides per minute. The actual digestion rate will vary depending on the source and batch of exonuclease III as well as the source of the DNA substrate, e.g., Guo et al., Nucl. Acids Res., Vol. 10 pg. 2065. Some experimentation may be necessary to obtain labeled stands of the desired length. Exonuclease III is available commercially, e.g., Boehringer Mannmheim (Indianapolis, IN), or Promega Biotec (Madison, WI). Also, T4 polymerase (BRL, Bethesda, MD) can be used for both the exonuclease and resynthesis steps.

The exonuclease treated restriction fragments serve as primer/templates for a DNA polymerase which re-synthesizes the digested strands in the presence of labeled precursors. The preferred labeled precursor is biotinylated uracil, as a substitute for thymidine. Re-synthesis is accomplished using DNA polymerase I or T4 DNA polymerase following the procedure of Langer et al., Proc. Nat'l. Acad. Sci., Vol. 78, pp. 6633-6637 (1981) which, in turn, is an adaption of the basic nick translation technique disclosed by Rigby et al., J. Mol. Biol., Vol. 113, pg. 237 (1977), e.g., 1 unit E. Coli polymerase I per microgram of DNA is incubated at 37°C in a solution consisting of 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM dithiothreitol, and 50 mM KCl. Also included in the solution are appropriate amounts of the triphosphate precursors (one or more of which are labeled), e.g., 50-100 micromolar of each for 20-50 micrograms per milliliter of restriction fragments. Under these conditions resynthesis is completed in about 40-60 minutes.

The labeled restriction fragments are broken into smaller fragments to ensure that the labeled regions on either end of the labeled restriction fragments are separated. (Otherwise, the labeled fragment on one end would be a part of a larger piece of single stranded DNA which contained complementary regions to the labeled fragment on the other end). Such breaking into smaller fragments is accomplished by any number of standard techniques, e.g. sonication, enzymatic treatment, or the like, Maniatis, et al. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, 1982).

After the labeled restriction fragments are appropriately broken into smaller pieces, they are denatured and single stranded labeled fragments are separated from unlabeled fragments. The separation can be accomplished in several ways. Whenever the preferred label, biotin, is used the preferred separation means is by way of a standard avidin affinity column, e.g. Bayer and Wilchek, "The Use of the Avidin-Biotin Complex as a Tool in Molecular Biology," Methods of Biochemical Analysis, Vol 26, pp. 1-45 (1980); and Manning et al., Biochemistry, Vol. 16, pp. 1364-1370 (1977). Avidin can be covalently coupled to a number of different substrates, such as glass, Sepharose, agarose, and the like, with standard techniques as described in the above references. Accordingly, Manning et al. and Bayer and Wilchek, pp. 9-16, are incorporated by reference. Avidin affinity columns are also available commercially, e.g. Zymed Laboratories, Inc. (South San Francisco, CA). The biotinylated probes are removed from the avidin column following the procedure of Chollet and Kawashima, Nucleic Acids Resources, Vol. 5, pp. 1529-1541 (1985).

Alternatives to the above labeling procedure are available. For example, after the DNA to be used in the probes is treated with a restriction endonuclease, the resulting restriction fragments are separated into two portions. The first portion undergoes treatment as described above. That is, it is treated with exonuclease to form template /primers for resynthesizing a labeled strand of DNA. The resulting resynthesized restriction fragments are then broken into smaller pieces, as described above. The label in this case, need not be biotin. For example, a radioactive label can be used. The second portion is also treated with an exonuclease, preferably exonuclease III. However, the reaction is allowed to proceed to completion so that each restriction fragment is converted into two noncomplementary single stranded pieces approximately half the length of the parent strand. These resulting single strands are then covalently linked to DBM paper using standard techniques, e.g. Maniatis et al; Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, 1982) pp. 335-339; and Alwine et al., Methods in Enzymology, Vol. 68, pp. 220-242 (Academic Press, New York, 1979). Accordingly, the cited pages of these references are incorporated by reference. The fragments of the first portion are denatured and combined with the DBM paper containing the covalently linked fragments of the second portion. Conditions are adjusted to permit hybridization of the labeled strands to complementary strands covalently linked to the DBM paper. The unlabeled strands from the first portion are washed from the paper (there being no complementary strands for them to hybridize to). After the washing the labeled strands are removed by heating, for example, and are ready for use.

Before application of the probe to the target DNA, the target DNA is treated with the same restriction endonuclease as was used in construction of the probe. After restriction endonuclease treatment the target DNA is treated with an exonuclease, preferably exonuclease-III or-T4-polymerase. Preferably, the conditions of exonuclease treatment are adjusted so that the lengths of single stranded regions created are substantially the same as the lengths of the probe DNA.

Hybridization of probe to target DNA is carried out using standard procedures, e.g. Gall and Pardue, Methods in Enzymology, Vol. 21, pp. 270-480 (1981); Henderson, International Review of Cytology, Vol. 76, pp. 1-46 (1982); and Angerer et al., in Genetic Engineering: Principles and Methods, Setlow and Hollaender, Eds., Vol. 7, pp. 43-65 (Plenum Press, New York, 1985). Accordingly, these references are incorporated by reference as guides for the use of the

invention in *in situ* hybridization. Briefly, probe prepared in accordance with the invention is combined with several other agents for reducing nonspecific binding, for maintaining the integrity of the biological structure being probed, and the like. The resulting mixture is referred to herein as the hybridization mix. Below, the method is applied in the Chromosome-specific staining of human chromosome 21.

5 Hind III restriction fragments of human chromosome 21 are available from the National Laboratory Gene Library Project through the American Type Culture Collection, Rockville, MD, Van Dilla et al., "Human Chromosome-Specific DNA Libraries: Construction and Availability," Biotechnology, Vol. 4, pp. 537-552 (1986). Alternately, such fragments can be produced following the disclosures in Van Dilla et al., cited above, or Fuscoe et al., "Construction of Fifteen Human Chromosome-Specific DNA Libraries from Flow-Purified Chromosomes," Cytogenet Cell Genet., 43: 79-86 (1986).

10 Clones from the library having unique sequence inserts are isolated by the method of Benton and Davis, Science, Vol. 196, pp. 180-182 (1977). Briefly, about 1000 recombinant phage are isolated at random from the chromosome 21-specific library. These are transferred to nitrocellulose and probed with nick translated total genomic human DNA.

15 Of the clones which do not show strong hybridization, approximately 300 are picked which contain apparent unique sequence DNA. After the selected clones are amplified, the chromosome 21 insert in each clone is ^{32}p labeled and hybridized to Southern blots of human genomic DNA digested with the same enzyme used to construct the chromosome 21 library, i.e., Hind III. Unique sequence containing clones are recognized as those that produce a single band during Southern analysis. Roughly, 100 such clones are selected for the heterogeneous mixture of probe DNA. The unique sequence clones are amplified, the inserts are removed by Hind III digestions, and the inserts are separated from the 20 phage arms by gel electrophoresis. The probe DNA fragments (i.e., the unique sequence inserts) are removed from the gel and treated with exonuclease III as described above, followed by resynthesis in the presence of biotinylated UTP precursor. The resulting double stranded fragments are sonicated so that on an average each fragment receives about 1.5-2.0 breaks. The resulting pieces are denatured and the biotinylated fragments are isolated by avidin affinity chromatography as described above.

25 Human lymphocyte chromosomes are prepared following Harper et al., Proc. Nat'l Acad. Sci., Vol. 78, pp. 4458-4460 (1981). Metaphase and interphase cells are washed 3 times in phosphate buffered saline, fixed in methanol-acetic acid (3: 1) and dropped onto cleaned microscope slides. Slides are stored in a nitrogen atmosphere at 20°C.

30 Slides carrying interphase cells and/or metaphase spreads are removed from the nitrogen treated with RNase (100 micrograms/ml for 1 hour at 37°C), treated for about 1-16 hours with Hind III at 37°C (10 units M 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, and 14 mM dithioerythritol at pH 7.6), treated with exonuclease III as described above, and dehydrated in an ethanol series. They are then treated with proteinase K (60 ng/ml at 37°C for 7.5 minutes) and dehydrated. The proteinase K concentration is adjusted depending on the cell type and enzyme lot so that almost no phase microscopic image of the chromosomes remains on the dry slide. The hybridization mix consists of (final concentrations) 2X SSC (0.15 M NaCl and 0.015 M sodium nitrate) 10 percent dextran sulfate, 500 micrograms/ml carrier DNA (sonicated herring sperm DNA), and 2.0 microgram/ml biotin-labeled probe DNA. This mixture is applied to the slides at a density of 3 microliters/cm² under a glass coverslip and sealed with rubber cement. After overnight incubation at 37°C, the slides are washed at 45°C (50% formamide-2XSS pH 7, 3 times 3 minutes; followed by 2XSSC pH 7, 5 times 2 minutes) and immersed in BN buffer (0.1 M Na bicarbonate, 0.05 percent NP-40, pH 8). The slides are never allowed to dry after this point.

40 The slides are removed from the BN buffer and blocked for 5 minutes at room temperature with BN buffer containing 5% non-fat dry milk (Carnation) and 0.92% Na Azide (5 microliter/cm² under plastic coverslips). The coverslips are removed, and excess liquid briefly drained and fluorescein-avidin DCS (3 microgram/ml in BN buffer with 5% milk and 0.02% Na Azide) is applied (5 microliter/cm²). The same coverslips are replaced and the slides incubated 20 minutes at 37°C. The slides are then washed 3 times for 2 minutes each in BN buffer at 45°C. The intensity of biotin-linked 45 fluorescence is amplified by adding a layer of biotinylated goat anti-avidin antibody (5 microgram/ml in BN buffer with 5% goat serum and 0.02% NaAzide) followed, after washing as above, by another layer of fluorescein-avidin DCS. Fluorescein-avidin DCS, goat antiavidin and goat serum are all available commercially, e.g., Vector Laboratories (Burlingame, CA). After washing in BN, a fluorescence antifade solution, p-phenylenediamine (1.5 microliter/cm² of coverslip) is added before observation. It is important to keep this layer thin for optimum microscopic imaging. This antifade significantly reduced fluorescein fading and allows continuous microscopic observation for up to 5 minutes. The DNA counterstains (DAPI or propidium iodide) are included in the antifade at 0.25-0.5 microgram/ml.

50 The red-fluorescing DNA-specific dye-propidium iodide (PI) is used to allow simultaneous observation of hybridized probe and total DNA. The fluorescein and PI are excited at 450-490 nm (Zeiss filter combination 487709). Increasing the excitation wavelength to 546 nm (Zeiss filter combination 487715) allows observation of the PI only. DAPI, a blue fluorescent DNA-specific stain excited in the ultraviolet (Zeiss filter combination 487701), is used as the counterstain when biotin-labeled and total DNA are observed separately. Metaphase chromosomes 21s are detected by randomly located spots of yellow distributed over the body of the chromosome.

55 The descriptions of the foregoing embodiments of the invention have been presented for purpose of illustration

and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

The complexity of the probes used in the present invention may be from about 50 kb to about 1 megabase or to about 750 kb, or preferably from about 200 kb to about 400 kb.

Test kits may comprise these probes.

When the probe nucleic acid sequences are propagated in a cell line and/or in one or more vectors, the cell line may be a hybrid cell line and the one or more vectors be selected from the group consisting of yeast artificial chromosomes, plasmids, bacteriophages and cosmids.

The hybrid cell line may be a human/rodent hybrid cell line.

When the probe nucleic acid sequences prior to hybridization to the targeted chromosomal material are broken into fragments of from about 200 bases to about 2000 bases, the size of the fragments may be about 1 kb.

When the size of the fragments is from about 800 bases to about 1000 bases and, when the hybridization is performed at a temperature of about 30°C to about 45°C, and when the subsequent washing steps are performed at a temperature of from about 40°C to about 50°C, the hybridization may be performed at a temperature of from about 35°C to about 40°C, or may be performed at a temperature of about 37°C, and the subsequent washing steps being performed at a temperature of about 45°C.

The labelled fragments may be detected after hybridization by flow cytometry, by microscopy (which may be automated) or by light scattering.

In the aspect of the invention wherein the targeted chromosomal material is of fetal cells that have been separated from maternal blood, said fetal cells may be separated from maternal blood by using monoclonal antibodies specific for said fetal cells, and the fetal cells may be leukocytes and cytotrophoblasts.

Such probes may comprise a prenatal screening test kit.

When high complexity nucleic acid probes which are substantially free of shared repetitive sequences are produced by a process incorporating a polymerase chain reaction (PCR) procedure, during said PCR process, sequences which are complementary to said shared repetitive sequences, and which have extended non-complementary ends or which are terminated in nucleotides which do not permit extension by a polymerase, may be hybridized to said shared repetitive sequences to inhibit amplification of such sequences.

Claims

1. A method of staining targeted chromosomal material based upon nucleic acid sequence to detect in an interphase cell one or more genetic translocations identified with chromosomal abnormalities, the method being performed outside the human body and comprising the steps of:

(a) hybridizing *in situ* a heterogeneous mixture of two or more human genome nucleic acid probes having a combined complexity of at least 40kb, which probes contain nucleic acid sequences which are substantially complementary to nucleic acid sequences that flank and/or extend partially or fully across breakpoint regions known to be associated with genetic rearrangements, wherein each probe is labelled with a different colour fluorochrome, with the targeted chromosomal DNA; and

(b) observing the proximity or overlap of the regions stained by each probe thereby allowing detection of a translocation.

2. A method according to claim 1 wherein observing the overlap of regions stained by each probe includes determination of a colour signal other than that of a fluorochrome on one of the probes.

3. A method according to claim 1 or claim 2 wherein the genetic rearrangements are identified with CML and/or ALL.

4. A method according to claim 1 or claim 2 wherein the genetic rearrangements are identified with Burkitts lymphoma.

Patentansprüche

1. Verfahren zum Färben von chromosomalem Ziel-Material basierend auf der Nucleinsäuresequenz, um in einer Interphasenzelle eine oder mehrere genetische Translokationen, welche mit chromosomal Anomalien identifi-

ziert werden, nachzuweisen, wobei das Verfahren außerhalb des menschlichen Körpers durchgeführt wird und die Schritte

- 5 a) in-situ-Hybridisieren eines heterogenen Gemisches zweier oder mehrerer Nucleinsäuresonden für das menschliche Genom, die eine kombinierte Komplexität von mindestens 40 kB aufweisen, welche Sonden Nucleinsäuresequenzen enthalten, die im wesentlichen komplementär zu Nucleinsäuresequenzen sind, welche Bruchstellenregionen, von denen bekannt ist, daß sie mit genetischen Umordnungen assoziiert sind, flankieren und/oder sich teilweise oder völlig darüber erstrecken, wobei jede Sonde mit einem Fluorochrom unterschiedlicher Farbe markiert ist, mit der chromosomal Ziel-DNA und
- 10 b) Beobachten der Nachbarschaft oder des Überlappens der durch jede Sonde gefärbten Regionen, wodurch der Nachweis einer Translokation ermöglicht wird, umfaßt.
- 15 2. Verfahren nach Anspruch 1, wobei das Beobachten des Überlappens der durch jede Sonde gefärbten Regionen die Bestimmung eines anderen Farbsignals als das eines Fluorochroms auf einer der Sonden beinhaltet.
- 20 3. Verfahren nach Anspruch 1 oder Anspruch 2, wobei die genetischen Umordnungen als CML und/oder ALL identifiziert werden.
- 25 4. Verfahren nach Anspruch 1 oder Anspruch 2, wobei die genetischen Umordnungen als Burkitt-Lymphom identifiziert werden.

Revendications

- 25 1. Procédé de coloration de matériel chromosomique cible basé sur la séquence d'acides nucléiques pour détecter dans une cellule en interphase une ou plusieurs translocations génétiques identifiées avec les anomalies chromosomiques, le procédé étant mis en oeuvre à l'extérieur du corps humain et comprenant les étapes qui consistent à:
 - 30 (a) hybrider *in situ* un mélange hétérogène de deux ou plusieurs sondes d'acides nucléiques du génome humain ayant une complexité combinée d'au moins 40 kb, lesdites sondes contenant des séquences d'acides nucléiques qui sont sensiblement complémentaires aux séquences d'acides nucléiques qui encadrent et/ou s'étendent partiellement ou complètement à travers les régions des points de cassure connues comme étant associées à des réarrangements génétiques, chaque sonde étant marquée à l'aide d'un fluorochrome de couleur différente, avec l'ADN chromosomal cible; et
 - 35 (b) observer la proximité ou le chevauchement des régions colorées par chaque sonde en permettant ainsi la détection d'une translocation.
- 40 2. Procédé selon la revendication 1, dans lequel l'observation du chevauchement des régions colorées par chaque sonde comprend la détermination d'un signal de couleur autre que celui d'un fluorochrome sur l'une des sondes.
- 45 3. Procédé selon la revendication 1 ou la revendication 2, dans lequel les réarrangements génétiques sont identifiés avec la LMC et/ou la LAL.
- 50 4. Procédé selon la revendication 1 ou la revendication 2, dans lequel les réarrangements génétiques sont identifiés avec le lymphome de Burkitt.



FIG. 1A

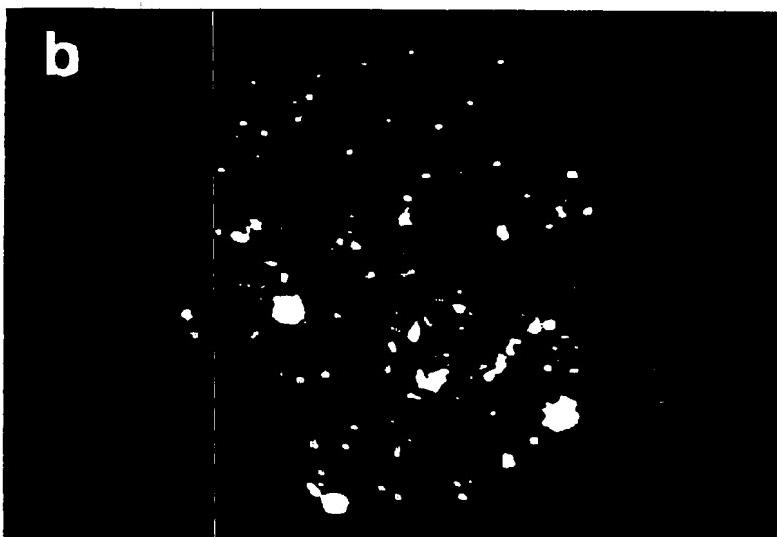


FIG. 1B

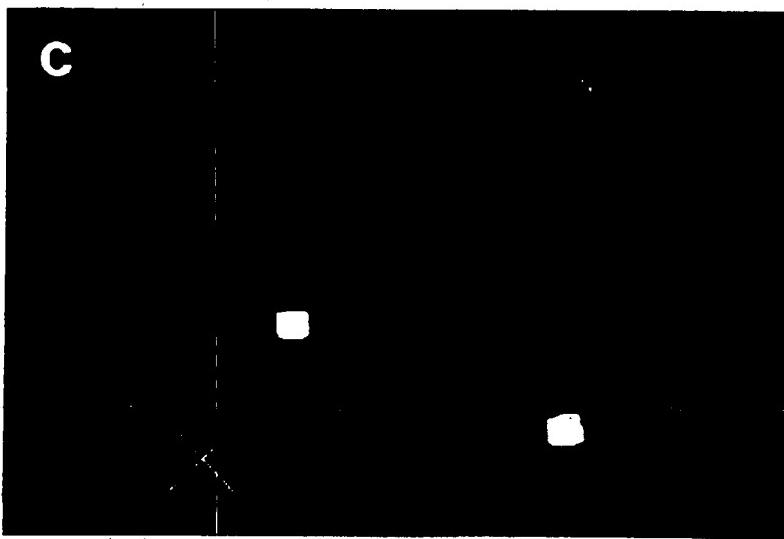


FIG. 1C

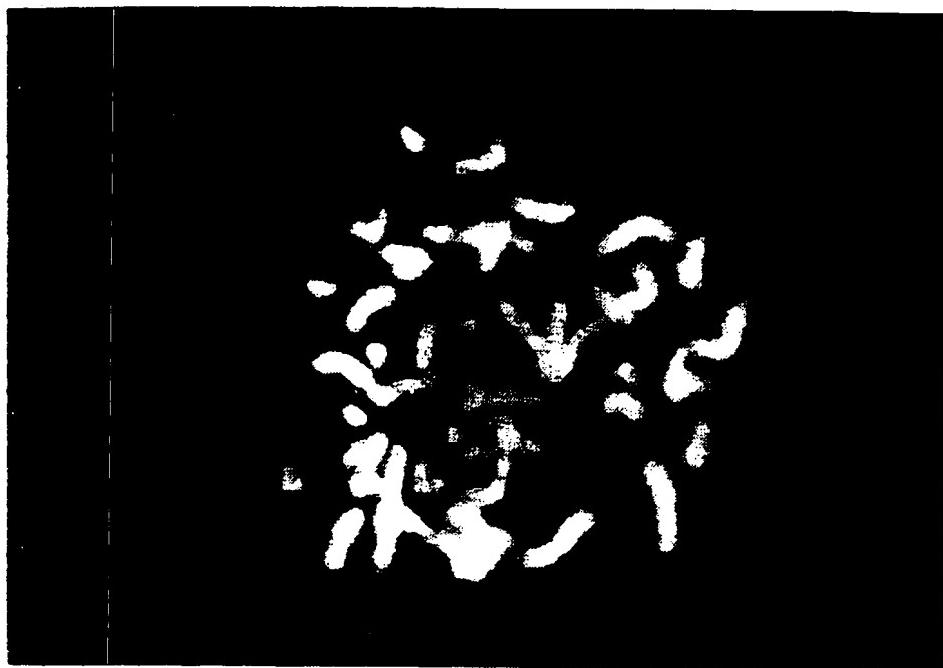


FIG. 2A



FIG. 2B



FIG. 3



**FIG.
4A**



**FIG.
4B**



**FIG.
4C**



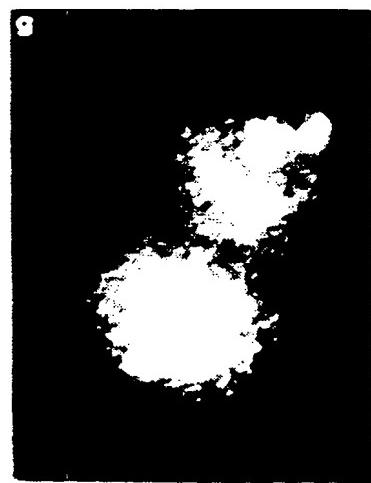
**FIG.
4 D**



**FIG.
4 E**



**FIG.
4 F**



**FIG.
4 G**



**FIG.
4 H**

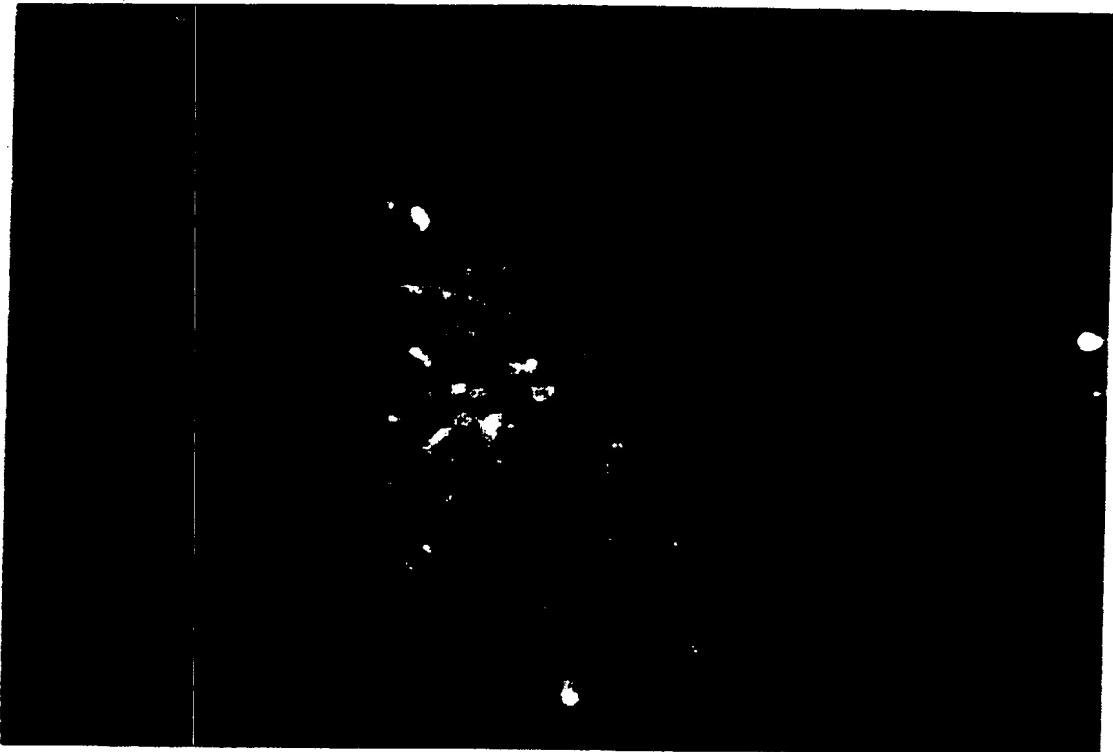


FIG. 5

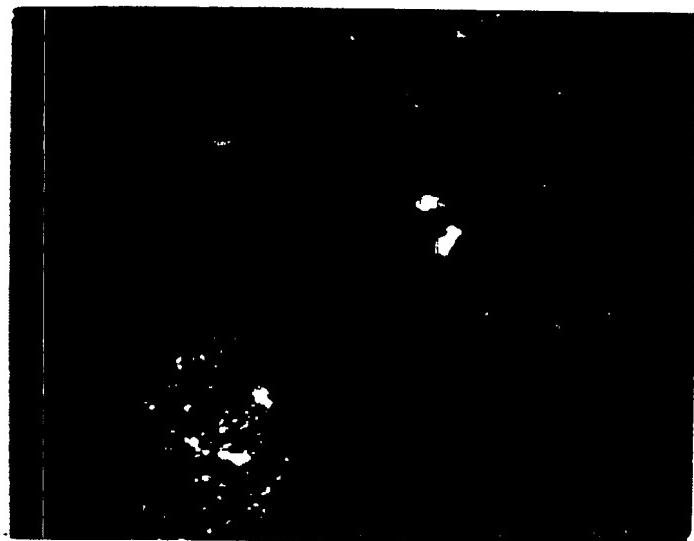


FIG. 6

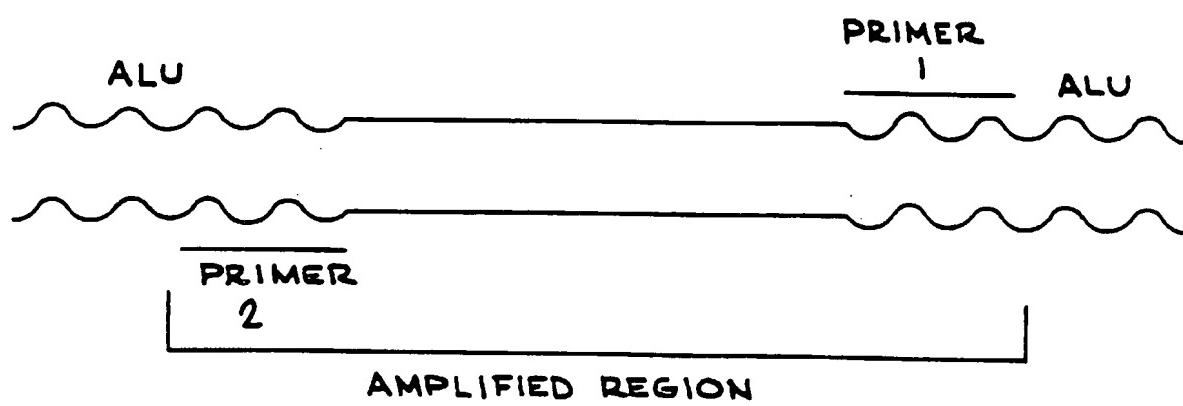


FIG. 7

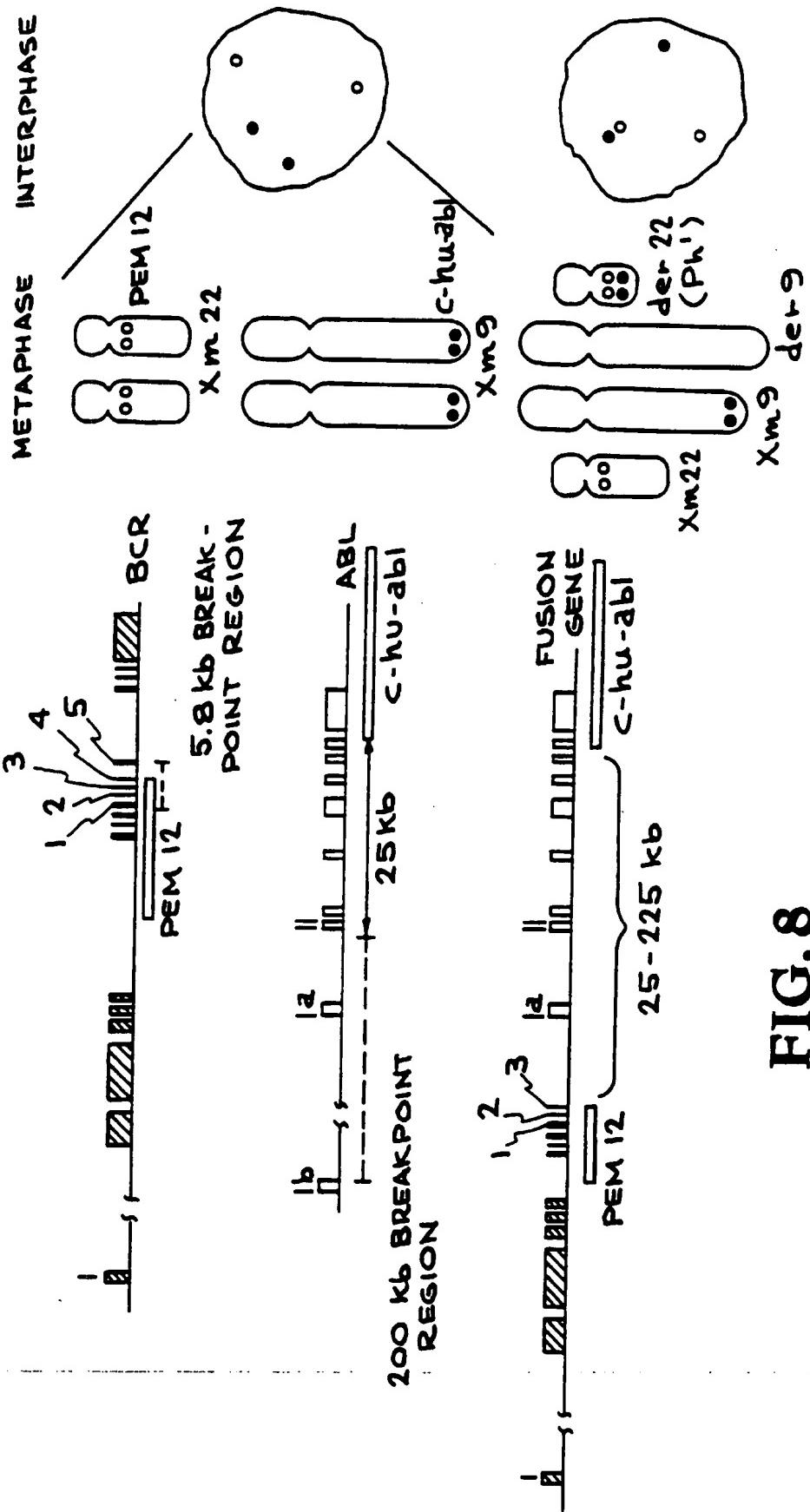


FIG. 8



FIG. 9A

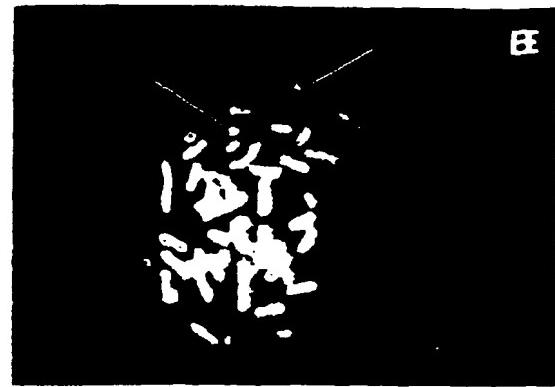


FIG. 9B

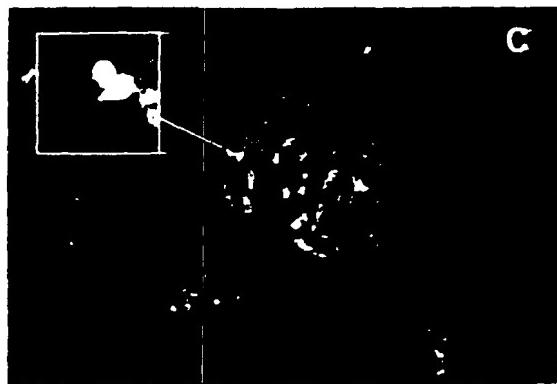


FIG. 9C

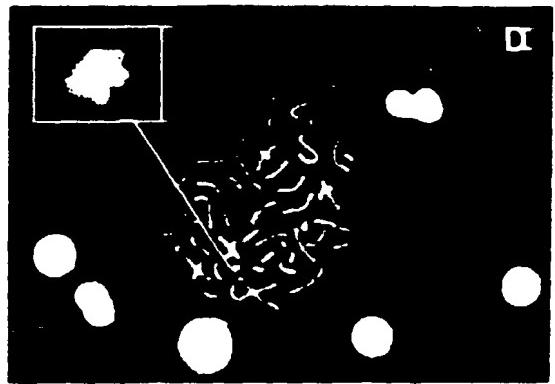


FIG. 9D

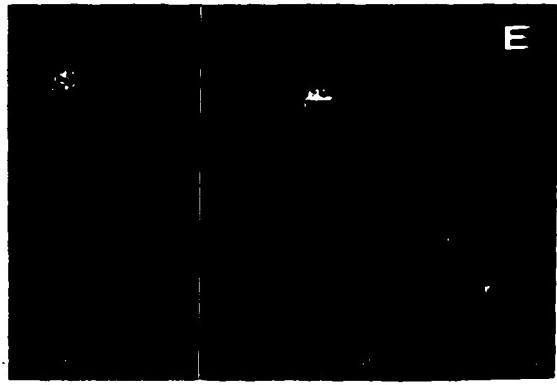


FIG. 9E



FIG. 9F

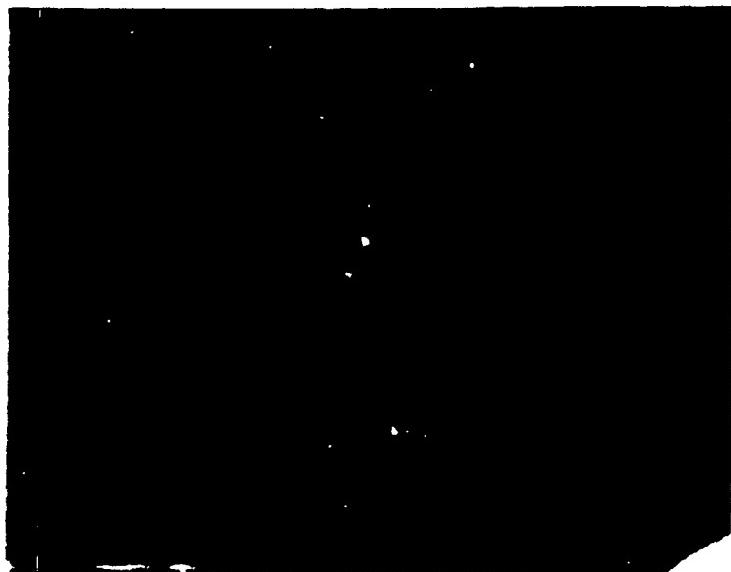


FIG. 10

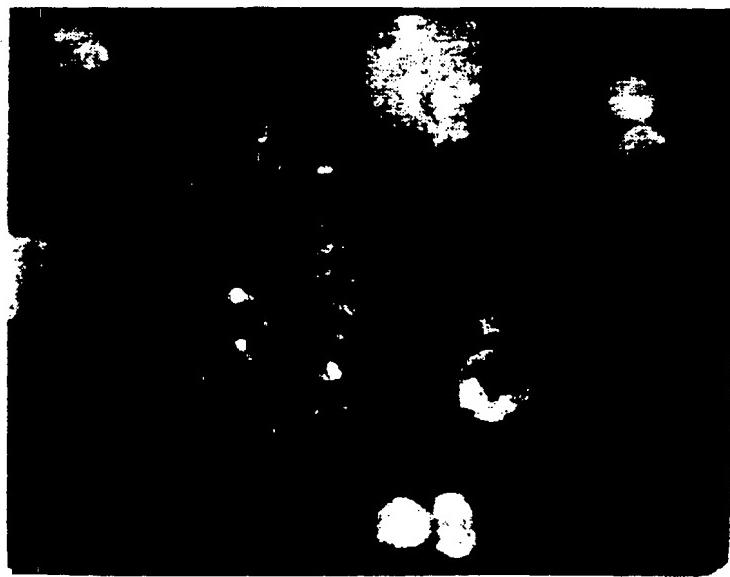


FIG. 12

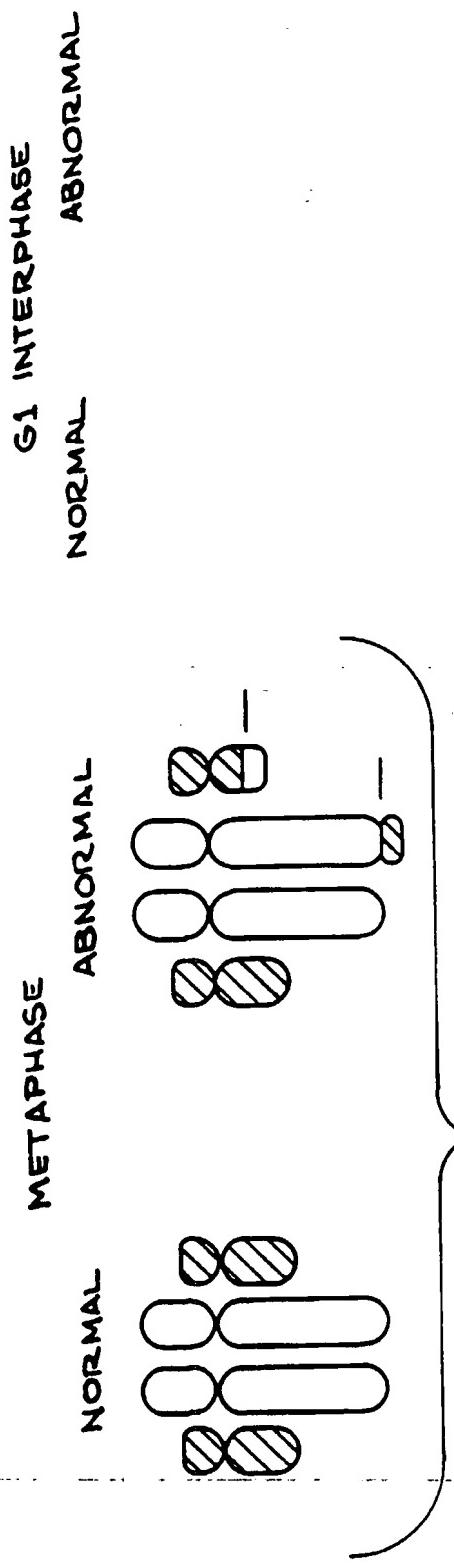


FIG. 11 A

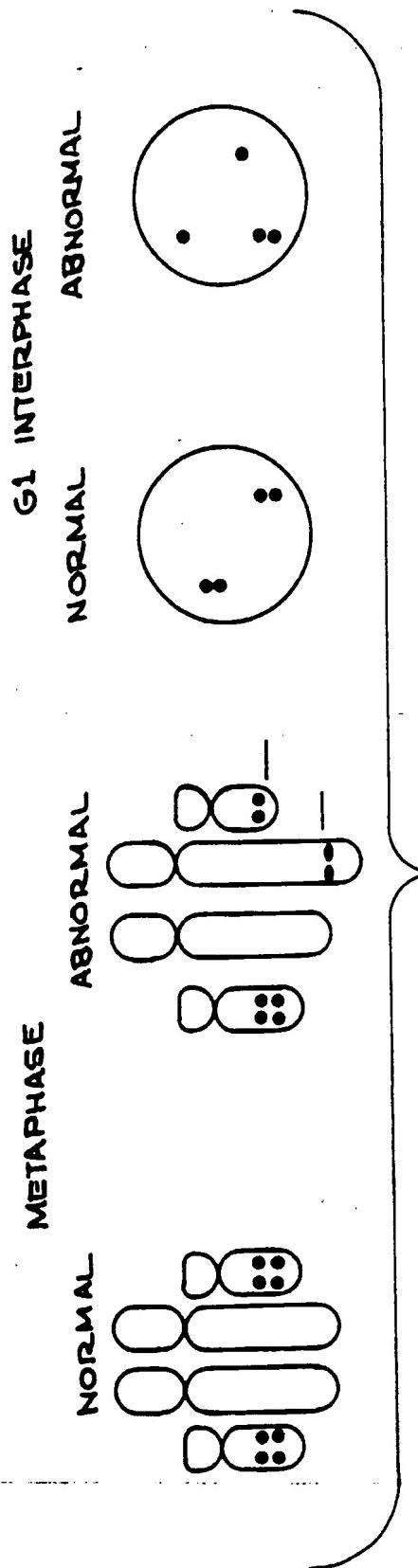


FIG. 11 B

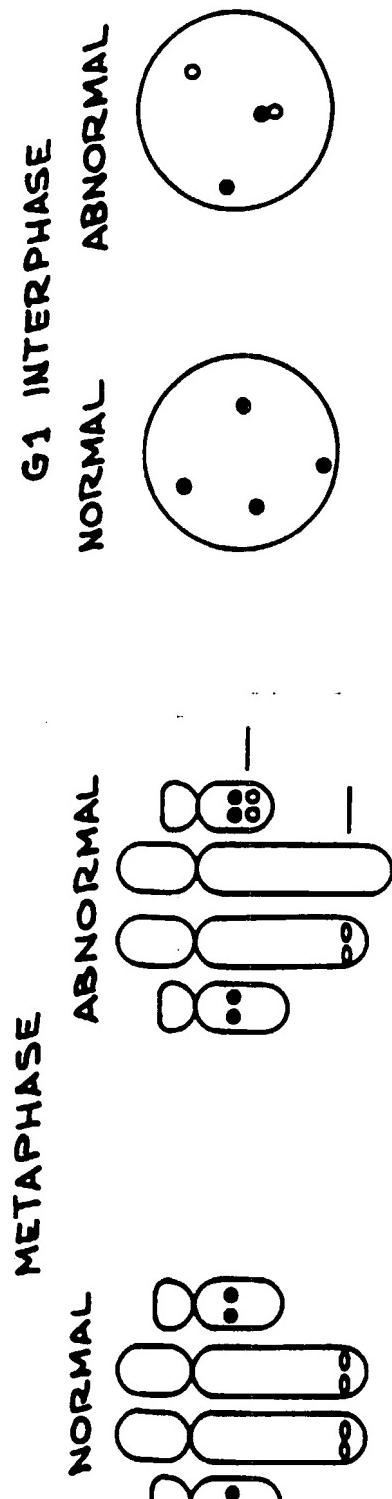


FIG. 11C

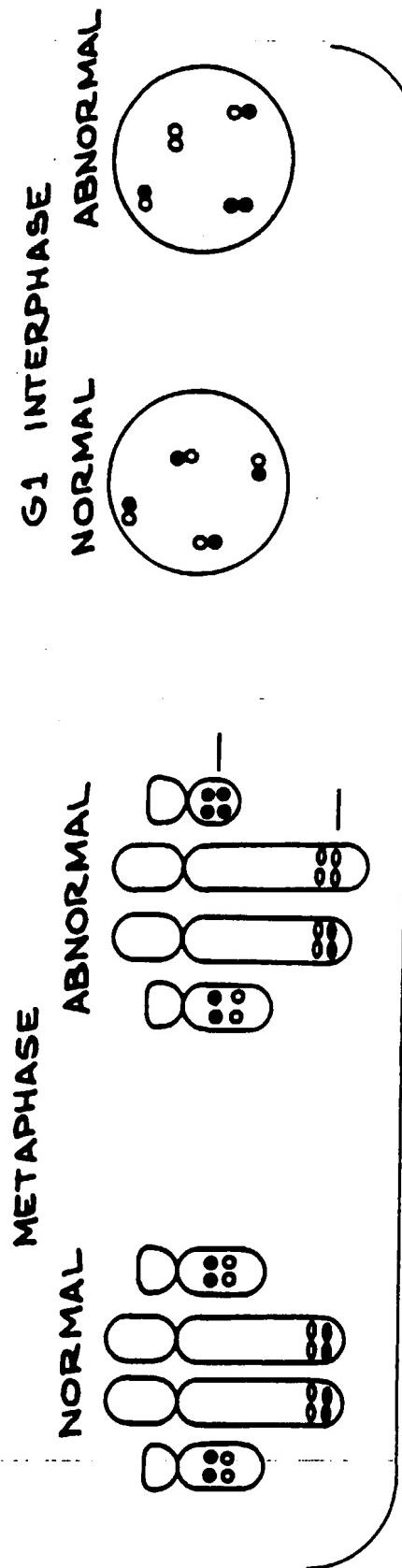


FIG. 11D

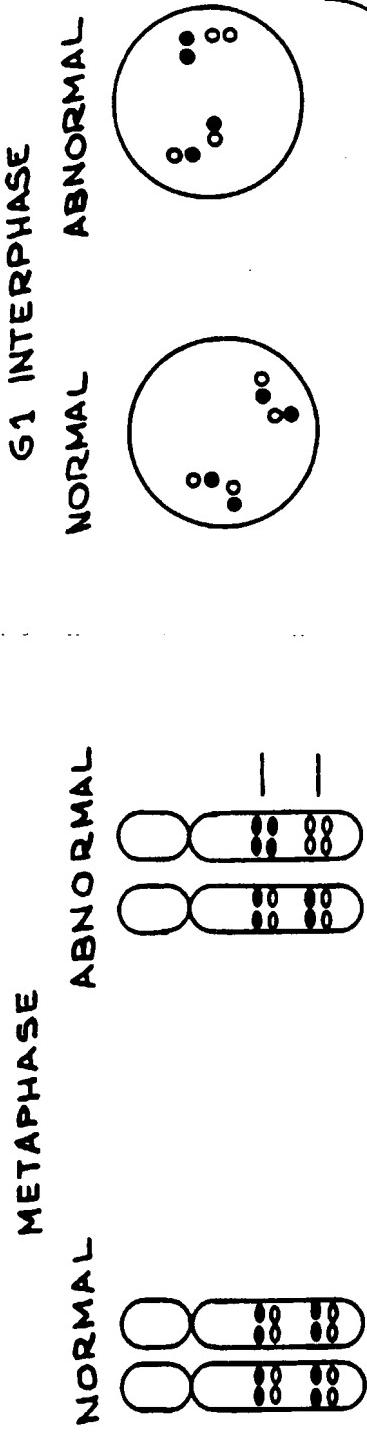


FIG. 11E

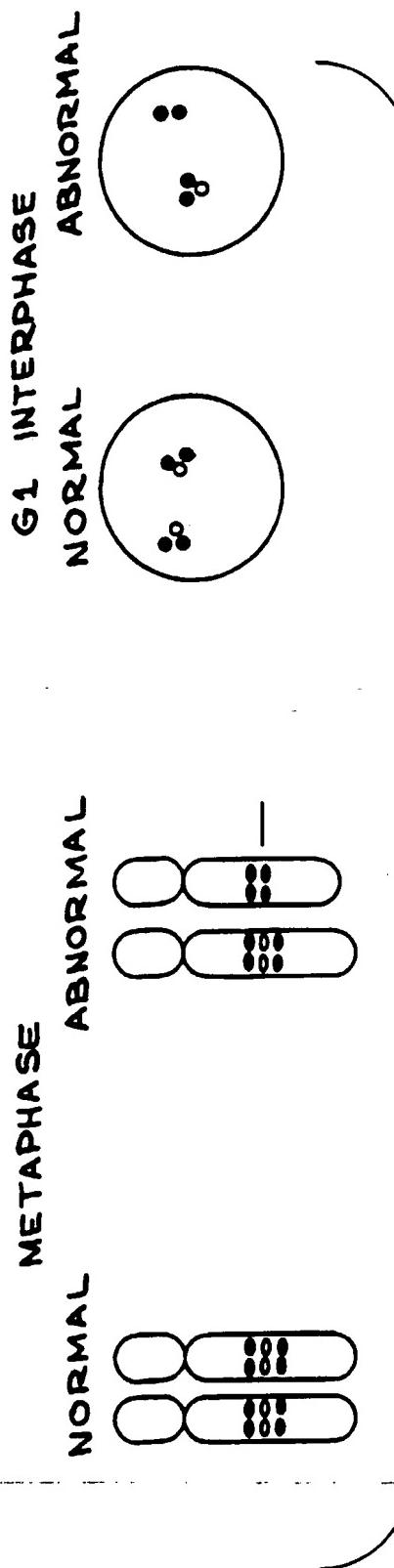


FIG. 11F

(19)



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• **Proc Natl Acad Sci USA 83 (1986) 2934**

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- | | |
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| <ul style="list-style-type: none">• Molecular Cloning, Sambrook et al, 2nd Edition (1989) p9.49• Hum Genet 77 (1987) 366• Hum Genet 67 (1984) 317• Hum Genet 72 (1986) 86• Proc Natl Acad Sci USA (9186) 4408 | <ul style="list-style-type: none">• Workshop Schedule for XIVth International Congress of Genetics• Exp Cell Res 153 (1984) 61• Cell 52 (1988) 51 |
|---|---|
-

Description**Background**

5 [0001] Chromosome banding techniques have facilitated the identification of specific human chromosomes and presently provide the major basis upon which chromosomal aberrations are diagnosed. The interpretation of chromosome banding patterns requires skilled personnel and is often technically difficult, especially with respect to detecting minor structural changes and when analyzing complex karyotypes, such as those of highly aneuploid tumor cells. An additional complexity is that readable metaphase chromosome spreads are sometimes very difficult or impossible to
 10 prepare from certain cell types or tissues. Alternative methods for identifying chromosomal aberrations would be valuable because they could augment current methods of cytogenetic analysis, particularly if such alternative methods were applicable to both mitotic and interphase cell populations.

[0002] Over the past few years, a considerable body of evidence has been obtained which indicates that the DNA of individual chromosomes occupy focal territories, or spatially cohesive domains, within mammalian interphase nuclei.
 15 Cremer, T. et al., *Hum. Genet.*, 60:66-56 (1982); Hens, L. et al., *Exp. Cell Res.*, 149:257-269 (1983); Schardin, M. et al., *Hum. Genet.*, 71:281-287 (1985); Manuelidis, L., *Hum. Genet.*, 71:288-293 (1985); and Pinkel, D. et al., *Proc. Natl. Acad. Sci. USA*, 83:2934-2938 (1986). These observations suggest that chromosome-specific probe sets could be used to detect numerical or structural aberrations of chromosomal domains in non-mitotic cells, an approach termed "interphase cytogenetics". Cremer, T. et al., *Hum. Genet.*, 74:346-352 (1986). Indeed, recent *in situ* hybridization studies
 20 have demonstrated the prenatal diagnosis of trisomy-18 with interphase cells and the detection of numerical chromosomal abnormalities in tumor cell lines using chromosome-specific repetitive DNAs as probes. Cremer, T. et al., *Hum. Genet.*, 74:346-352 (1986) and Cremer, T. et al., *Exp. Cell Res.*, 176:119-220 (1988). All chromosome-specific repetitive DNAs reported to date are localized to discrete subregions of each chromosome and, thus, such DNA probes are unsuitable for analyses of many types of chromosomal aberrations (e.g., translocations and deletions). If it were possible
 25 to detect uniquely the spectrum of sequences comprising a specific chromosome, analysis of aberrations of chromosomal domains in non-mitotic cells would be possible. Furthermore, such a general labeling technique would make it possible to address fundamental questions concerning the spatial organization of chromosomal DNA within interphase nuclei.

30 Disclosure of the Invention

[0003] The subject invention relates to a method of detecting, identifying and/or quantitating selected individual chromosomes in mammalian mitotic or interphase cells, by means of chromosomal *in situ* suppression (CISS) hybridization and its use in analyzing cells for the occurrence of chromosomes, chromosome fragments, or chromosome aberrations, such as those associated with a condition or disease. In the method of the present invention, chromosome-specific probes (DNA or RNA) are combined with a sample to be analyzed, in such a manner that an individual chromosome(s) of interest is labeled and the complex spectrum of sequences which comprise the chromosome can be detected. The probes used in the present method are of high genetic complexity and can be appropriately-selected cloned DNA or RNA fragments, used individually or in pools, or chromosome library DNA.

[0004] The method of the present invention, referred to as CISS hybridization, is particularly useful because it can be used to specifically stain individual mammalian chromosomes at any point in the cell cycle. It can be used to assess chromosomal content, particularly chromosome aberrations (e.g., deletions, rearrangements, change in chromosome number) which, until the present invention, it has been time-consuming and/or difficult, if not impossible, to detect. The method is useful in providing a rapid and highly specific assessment of individual mammalian chromosomes in any context (e.g., diagnosis and/or monitoring of a genetic condition or a disease state) in which such an assessment is desired.

Brief Description of the Drawings**50 [0005]**

Figure 1 presents an outline of the CISS hybridization method of the present invention.

Figure 2 shows suppression from cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations of human competitor DNA. Biotin-labeled chromosome 7 DNA inserts (20 µg/ml) were prehybridized for 20 minutes with human genomic DNA prior to hybridization with metaphase chromosome spreads and detection with FITC-labeled avidin. Human DNA concentrations were: A, 0 µg/ml; B, 50 µg/ml; C, 100 µg/ml; D, 200 µg/ml; E, 1000 µg/ml; F, same metaphase spread as in E post-stained with DAPI. Genomic salmon DNA was added to each sample to adjust the final DNA concentration to 1.0 mg/ml (see the text for details). The arrows mark the

target chromosome 7 and the arrowheads mark additional strong signals on non-7 chromosomes. All negatives printed were exposed and developed under identical photographic conditions.

Figure 3 shows the effect of pre-annealing time on the specificity and strength of the hybridization signal. Biotin-labeled chromosome 7 DNA inserts (20 µg/ml) were preannealed with 200 µg/ml human competitor DNA for the following times, prior to hybridization to metaphase A, 0 minutes; B, 2 minutes; C, 5 minutes; D, 20 minutes.

Figure 4 shows decoration of A chromosome 1, B chromosome 7, C chromosome 4, D chromosome 18, E chromosome 13 and F chromosome 20 in normal human lymphocytes. Only the chromosome 13 insert DNA pool shows significant cross-hybridization to other chromosomes after the prehybridization suppression step. The detection of chromosome 20 (F) was done with the entire chromosome library (including λ phage arms) and detected with avidin-alkaline phosphatase using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) as the enzyme substrate mixture. The signal of chromosome 1 (A) was amplified by the sandwich technique of Pinkel *et al.* (1986).

Figure 5 shows chromosome domains in human lymphocyte nuclei delineated by preannealed chromosome library DNA inserts. Hybridization to acetic acid-methanol fixed nuclei was detected by fluorescein isothiocyanate (FITC)-conjugated avidin (A-E) or alkaline phosphatase-conjugated avidin (F). Domains are shown for chromosome 1

(A,B), chromosome 7 (C,D) and chromosome 18 (E,F). A predominant staining of the centromere region is seen within the chromosome 7 domains, reflecting preferential hybridization of the chromosome 7-specific alphoid DNA repeat; a similar signal distribution on metaphase chromosomes was also observed in this particulate experiment.

Figure 6 shows chromosomal *in situ* suppression (CISS) hybridization of chromosome 1 inserts to metaphase spreads of glioma cell lines detected with avidin-FITC (A,C) and poststained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (B,D). A,B TC 620 show two apparently complete 1 chromosomes (small arrows in B) and two marker translocation chromosomes (arrowheads) specifically decorated by these inserts (A). One of the two marker chromosomes contains a 1p (lower left), the other a 1q arm (lower right); the 1p terminal (relatively GC rich region) in the two normal chromosomes and submetacentric marker is less completely delineated. Also, the 1q12

regions here show little decoration in contrast to most experiments. X 950. C,D Typical TC 593 metaphase spreads show six specifically decorated chromosomes. Three acrocentric marker chromosomes all with truncation of 1p

show particularly intense fluorescence of repeats that localize to 1q12 (arrows in C). In two of these, 1q arms appear to be complete, while a major deletion is obvious in the third (the arrow in D). A fourth decorated chromosome (small arrowhead in C,D) again shows a major deletion of the distal part of 1q, but has retained an apparently complete 1p arm. A fifth submetacentric chromosome (large arrowhead) contains an apparently complete 1p arm; the DNA of its short arm has not been identified. Note the similarity of this marker to one of the marker chromosomes of TC 620 (1p) described above. The sixth entirely decorated chromosome is an iso (1p) as demonstrated by DAPI-banding (open arrows). X 1200

Figure 7 shows CISS hybridization of chromosome 4 library inserts detected with avidin-FITC; interphase nuclei of TC 593 (A-C) and TC 620 (D). Note that the two apparently complete interphase domains are close to each other

in B, but widely separated in A and C. TC 620 interphase nucleus (D) shows four chromosome 4 interphase domains of largely different sizes. Metaphase spread of TC 593 (E) shows two apparently complete 4 chromosomes, and a small decorated region (arrow) in a submetacentric chromosome. This marker with translocated 4

sequences was observed in about 30% of the spreads. TC 620 metaphase spread (F) shows one apparently complete chromosome 4 and three translocation markers (t) containing different amounts of chromosome 4 material. G-J Double hybridization of biotinylated chromosome 7 inserts and an aminoacetylfluorene (AAF)-modified 7-specific alphoid repeat. G Chromosome 7 inserts depict five entirely decorated metaphase chromosomes. Four of them are complete 7 chromosomes, the fifth (arrow) is an iso (7p) (see Fig. 3E). H The same field as G showing AAF-7 alphoid signals on only four decorated chromosomes; no signal is detected on the iso (7p). I An interphase

nucleus of TC 593 shows five domains delineated by chromosome 7 inserts; four of these are labeled by 7 alphoid probes (J). The arrow in I represents the iso (7p) marker in interphase.

Figure 8 shows CISS hybridization of library inserts of chromosomes 7 and 18 to metaphase spreads of glioma cell lines detected with avidin-FITC. X 875. A,B TC 620 hybridized to chromosome 7 inserts. Three apparently normal 7 chromosomes and an additional translocation chromosome containing 7 sequences are indicated by large arrowhead in A; DAPI-stained complete chromosomes are indicated by the small arrows in B. Other studies (see the text)

indicated a translocation of 7pter-q11 in the marker chromosome (large arrowhead). D, The corresponding frame counterstained with DAPI. E, Metaphase spread from pseudotetraploid TC 593 shows five chromosomes highlighted by 7 library inserts. The metacentric chromosome (m) represents the iso (7p) marker typical for this line (see also Fig. 2G). Insert chromosomes (small arrows) show DAPI-stained normal and metacentric 7 chromosomes. The landmark band 7q21 and a block of constitutive heterochromatin at 7q11 are both prominent on the normal

chromosome 7 insert, (arrows) but not present on the marker chromosome. Instead both arms of the latter show a mirror-like staining pattern with a faint distal band at 7p21. F,G TC 593 hybridized to chromosome 18 inserts. Four decorated 18 chromosomes are shown in F and three of them in G by DAPI staining are clearly translocated (t).

Figure 9 is a summary chromosome idiogram of complete and aberrant chromosomes detected by CISS hybridization of library inserts of chromosomes 1, 4, 7, 18 and 20 in glioma cell lines TC 620 (left) and TC 593 (right). G-bands (black) are shown with approximate breakpoints suggested by our data; the shaded areas with a wavy pattern are from other chromosomes that constitute part of the marker translocation chromosomes. The black dot beside two of the TC 620 translocated 4 segments indicates that the assignment of the chromosome 4 material is based on circumstantial evidence (e.g., size measurements). A small translocation of chromosome 18 material in ca. 20% of TC 593 metaphase spreads (+) also could not be further identified. Note the over-representation of 7p in both cell lines.

Figure 10 shows representative prophase (A) and interphase nuclei (B-F) reflect metaphase abnormalities in glioma lines (cf. Figs. 1-3). A, Detection of the 7p translocation (t) in a prophase TC 620 nucleus. X 1,000. B, Five well-separate chromosome 7 domains detected in TC 593. X 1,240. C, Two large and one very small 18 domains (indicated by arrow head) in TC 620. X 1,450. D, Four chromosome 18 domains detected in TC 593; one of these signals (arrow) appears smaller. X 1,450. E, Four chromosomal 1 domains in TC 620 (cf. Fig. 1A, B). X 1,200. F, At least five chromosome 1 domains in TC 593; one (arrow head) is appreciably smaller than the others. X 1,250. G,H, CISS hybridization of a technically poor metaphase spread of TC 593 poststained with DAPI (G) still highlights four distinct chromosomes bearing 18 sequences (H). X 1,000.

Figure 11 is a graphic representation of the interphase and/or metaphase counts of chromosomes 7 (A-C), 22 (D) and 4 (E) by CISS hybridization. Interphase counts were performed on 150 nuclei of well-hybridized preparations. For metaphase counts > 25 complete DAPI-stained spreads were evaluated. A-C Counts of 7 specific alphoid repeats (white columns) compared to 7 library inserts (shaded columns): A, interphase nuclei of phytohemagglutinin-stimulated human lymphocytes (46,XY); B, TC 620 interphase nuclei (7-specific alphoid repeat) and metaphase spreads (7 library inserts); C, TC 593 interphase nuclei (7-specific alphoid repeat) and metaphase spreads (7 library inserts). High stringency hybridization (see Materials and methods) of 7 alphoid repeat was used to avoid cross-hybridization to other chromosomes. In cases of double hybridization with both 7 library inserts and alphoid repeat (shown in Fig. 2 G-I) standard conditions with 50% formamide were sufficient to avoid cross-hybridization, possibly due to the presence of human competitor DNA. D, Counts of chromosome 22 (library inserts) in metaphase spreads of TC 620 (black columns) and TC 593 (shaded columns). For comparison, CISS hybridization was simultaneously performed with 7 library inserts in these experiments as an internal control (see C,D and Fig. 7). E, Interphase counts (white columns) and metaphase counts (shaded columns) compared in TC 593 hybridized with chromosome 4 inserts. Note the ratio of signal preparations 2:3 are the same in metaphase and interphase.

Figure 12 shows a TC 620 metaphase spread after double hybridization with inserts from chromosomes 7 and 22 (both labeled with biotin and detected with avidin-FITC). Two strongly decorated 22 chromosomes (arrows), three complete 7 chromosomes, and the metacentric marker chromosome containing 7pter-q11 are also seen.

Figure 13 is a graphic representation of the relative size of decorated normal and aberrant chromosomes 4, 7 and 18 in typical metaphase spreads (n=24) from glioma cell lines TC 593 and TC 620. Individual areas were normalized so that a complete chromosome is represented by an area of 1 (see legend to Table 1). The total added signals reflect the number of specific chromosome equivalents present. The white regions correspond to apparently normal chromosomes, the black regions indicate small free chromosome segments entirely decorated by specific library inserts, and translocated segments are shaded. One of the three translocated 18 chromosomes in TC 593 represents a complete chromosome by this measurement (indicated by the black dot), while the two other translocations are slightly smaller, possibly due to the small sample size.

Figure 14 shows specific labeling of human chromosome 21 by CISS hybridization with biotinylated DNA probe sets. A, plasmid PPW519-1R (6-Kb insert) hybridized to a normal lymphocyte metaphase spread. Signals are located on the termini of 21q (see 4',6-diamidino-2-phenylindole (DAPI)-stained chromosomes in Inset) as verified by DAPI banding (not shown). B and C, Normal human lymphocyte metaphase (B) and nuclei (C) after hybridization with the 94 Kb plasmid pool probe set. The terminal band 21q22.3 is specifically labeled. D and E, Signals on trisomy 21 (47, + 21) lymphocyte metaphase spreads after hybridization with the 94 Kb probe set (D) or chromosome 21 library DNA inserts with the CISS hybridization (14) protocol. (E) Three chromosomes 21 are entirely delineated by the library inserts; additional minor signals (see text) are indicated by arrowheads (also in G). (F-J) Labeling of trisomy 21 lymphocyte nuclei by the library inserts (F and G; compare with E).

Detailed Description of the Invention

[0006] The present invention is based on a hybridization strategy in which suppression of hybridization signals from ubiquitous repeated DNA sequences is achieved by using total DNA in a reannealing procedure which is based on rapid reassociation kinetics. The hybridization method of the present invention referred to as chromosomal in situ suppression (CISS) hybridization because of the selective suppression of such signals, has been shown to result in specific cyto-staining of one or more selected individual chromosomes, particularly human chromosomes, at any point in the

cell cycle and has been used to detect, identify and quantitate chromosomal aberrations in both mitotic cells and interphase cells (i.e., interphase nuclei).

[0007] Described below and in greater detail in the Examples, are the following:

- 5 1. specific staining in mitotic and interphase cells of individual human chromosomes, by the method of the present invention (CISS hybridization), using chromosome-specific probe sets which are of high genetic complexity (i.e., chromosome library DNA, cloned DNA fragments);
- 10 2. specific staining of metaphase and interphase tumor cells by CISS, using chromosome-specific library probes; and
- 15 3. rapid detection in mitotic and interphase cells from a variety of sources of aberrations in a human chromosome (chromosome 21) which is associated with a genetic condition (Down syndrome), using CISS hybridization.
- 20 4. demonstration that a nested set of chromosome specific unique sequence probes has been used to identify chromosome aberrations and to detect genetic disease (e.g., Down Syndrome).

15 Specific Staining of Individual Human Chromosomes

[0008] By use of the CISS hybridization method, individual (such as the X and Y chromosomes or homolog pairs of chromosomes 1-22) human chromosomes have been specifically stained in both mitotic and interphase cells. This has been carried out in both metaphase spreads and interphase nuclei and has been used to stain or label one selected (individual) chromosome and to stain or label multiple selected (individual) chromosomes simultaneously, using, respectively, signal-probe CISS hybridization and multi-probe CISS hybridization in conjunction with an appropriate detection method. The method is represented schematically in Figure 1.

Specific Chromosome Staining Using Genomic DNA Libraries and Cloned DNA

[0009] CISS hybridization was carried out as follows, to produce specific staining of individual human chromosomes, using commercially-available genomic DNA libraries that originated from flow-cytometry sorted human chromosomes and cloned DNA fragments. Van Dilla, M.A. et al., *Biotechnology*, 4:537-552 (1986). Suppression of hybridization signals from ubiquitous repeated sequences, such as the Alu and KpnI elements, was achieved using total human DNA in a reannealing procedure that is based on rapid reassociation kinetics. Similar principles have been used by others to facilitate the selective hybridization of unique sequence subsets from cosmid DNA clones for Southern blotting and in situ hybridization experiments. Sealey, P.G. et al., *Nucleic Acids*, 13:1905-1922 (1985); and Landegent, J.E. et al., *Hum. Genet.*, 77:366-370 (1987). Specific labeling of individual chromosomes in both metaphase spreads and interphase nuclei, is carried out (and shown to have occurred) in the following manner, which is described in detail in the Examples. The feasibility of using computer-assisted optical sectioning for 3-D reconstruction of chromosomal domains for the analysis of nuclear topography was also demonstrated in conjunction with CISS hybridization.

[0010] Initially, genomic DNA from a selected chromosome or selected chromosomes is prepared for use as probe DNA. Genomic DNA is available from several sources. For example, one or more genomic DNA libraries, each containing the chromosome of interest (a chromosome-derived library), is used to produce the necessary DNA probes. Such libraries can be commercially-available genomic DNA libraries that originated from flow-cytometry sorted human chromosomes. These are available from the American Type Culture Collection (Rockville, MD). Such DNA libraries for human chromosomes 1, 4, 7, 8, 9, 12, 13, 14, 16, 17, 18, 20, 21, 22 and chromosome X have been used in the present method, as described in the Examples. Other commercially available genomic DNA libraries or genomic DNA libraries from noncommercial sources can also be used. Alternatively, individual plasmid, phage, yeast artificial chromosomes with non-yeast DNA inserts, and cosmid DNA clones can be used as a source of DNA probes for a selected individual chromosome or multiple selected chromosomes. In the case of DNA from a genomic library, the DNA can be separated as a pool from the vector containing it, prior to labeling with a detectable signal, or can be used without separation from the vector.

[0011] Probes are labeled with a detectable signal, which can be a fluorescent reporter, one member of a specific binding pair (e.g., biotin-avidin or ligand-antibody), or an enzyme. DNA removed from the vector is labeled by nick translation (using, for example, Bio-11-dUTP), by random primer extension with (e.g., 3' end tailing), for example, the Amersham multiprime DNA labeling system, substituting dTTP with Bio-11-dUTP, or other appropriate technique. In the case of DNA which has not been separated from the vector, biotin labeling is carried out directly by nick translation, using standard techniques. Brigati et al., *Virology*, 126:32-50 (1983). Other labels can be added in a similar manner (e.g., 2,4-dinitro phenol, digoxin).

[0012] Probe size is carefully selected and controlled in order to facilitate probe penetration and to optimize reannealing hybridization. Labeled DNA fragments smaller than 500 nucleotides are used, and, more generally, the majority of the probes are 150-250 nucleotides in length. Probes of this length are made from longer nucleotide sequences

using publicly available restriction enzymes or known techniques for producing and recovering appropriately-sized fragments. It is also possible, if the nucleotide sequence of a selected chromosome is known, to synthesize an oligonucleotide having that sequence, using known techniques. Such oligonucleotides, once labeled, can be used to decorate specific chromosomal regions. For example, oligonucleotide probes which specifically hybridize to telomeric sequences of mammalian chromosomes have been identified. Moyuif et al., *Proceedings of the National Academy of Sciences, USA*, Sept. 1988.

[0013] Competitor DNA, which is DNA which acts to suppress hybridization signals from ubiquitous repeated sequences, will be selected as needed (e.g., based on the mammal whose chromosomes are being analyzed). In the case of analysis of human chromosomes, competitor DNA is total human DNA which acts to suppress hybridization from ubiquitous repeated sequences, such as the *Alu* and the *KpnI* elements. It is available from many sources. For example, human genomic DNA from placenta or white blood cells can be prepared using known techniques, such as that described by Davis et al. Davis, L.G. et al., *Basic methods in molecular biology*, Elsevier, N.Y./ Amsterdam (1986). It is digested, using standard methods (e.g., with DNase), to produce competitor DNA fragments within the same size distribution as the probe DNA.

[0014] DNA from another source, which will compete with only a small portion of the human DNA and which is used, as necessary, to adjust the total (final) DNA concentration of the hybridization mixture will also be included, as needed. This DNA is referred to as carrier DNA. This DNA is produced or treated, using standard methods, so that it is within the same size distribution as the probe DNA.

[0015] Initially, probe DNA bearing a detectable label and competitor DNA are combined under conditions appropriate for preannealing to occur. The quantity of probe DNA combined with competitor DNA is adjusted to reflect the relative DNA content of the chromosome target. For example, chromosome 1 contains approximately 5.3 times as much DNA as is present in chromosome 21. Probe concentrations were 30 µg/ml and 5 µg/ml, respectively. When total genomic library DNA is used as the probe mixture (instead of purified DNA inserts), approximately 10 times as much labeled DNA is added to compensate for the vector sequences, which are present in large quantities. Only twice as much labeled library DNA is added in the case of the libraries LA0XNL01 (X chromosome) and LA16NL02 (chromosome 16) because the human DNA inserts constitute almost half of the total library DNA. Carrier DNA, such as trout or salmon testis DNA, is added to bring the total DNA concentration to a predetermined level, if necessary. As described herein, sufficient salmon testis DNA was added to result in a final DNA concentration of 1.0 mg/ml in the hybridization mixture (which includes all three types of DNA: probe DNA, competitor DNA and DNA which does not significantly compete).

[0016] The resulting hybridization mixture is treated (e.g., by heating) to denature the DNA present and incubated at approximately 37°C for sufficient time to promote partial reannealing.

[0017] The sample containing chromosome DNA to be identified (specifically labeled) is also treated to render DNA present in it available for hybridization with complementary sequences, such as by heating to denature the DNA. The hybridization mixture and the sample are combined, under conditions and for sufficient time conducive to hybridization. After sufficient time, detection of specific labeling of the chromosome target is carried out, using standard techniques. For example, as described in the Examples, a biotinylated probe is detected using fluorescein-labeled avidin or avidin-alkaline phosphatase complexes. For fluorochrome detection, samples are incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin DCS (see Example 1). Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat anti-avidin D antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples are incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in AP-buffer, as described in Example 1). The enzyme reaction is carried out in, for example, AP buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate and stopped by incubation in 2 X SSC.

45 Detection of Chromosome Aberrations Using CISS Hybridization

[0018] Using the above-described steps, it is possible to specifically stain or label any selected individual chromosome (or chromosomes) referred to as a target chromosome, or a subregion(s) thereof. As explained in the examples, the present method has been shown to be useful in a variety of cells, both in mitotic (e.g., metaphase, prophase) and interphase cells. As described in detail in Example 2, the CISS hybridization method of the present method is useful for rapidly screening mitotic and interphase aneuploid tumor cells for complex numerical and structural aberrations of individual chromosomes (e.g., changes in number of chromosomes, deletions and rearrangements or translocations).

[0019] In this context, biotinylated library DNA inserts were used in the CISS hybridization method to produce hybrid molecules which were detected using known techniques. Two glioma lines were used as general models of aneuploid cells, particularly tumor cells. One was an oligodendrogloma line and the other was a glioblastoma line. These were analyzed, using the biotinylated DNA probes specific for chromosome 1, 4, 7, 18 and 22. Specific labeling of the chromosomes, from pter to qter, made it possible to visualize numerical changes, deletions and rearrangements in

these chromosomes in metaphase spreads and in early prophase and interphase nuclei. Complete chromosomes, deleted chromosomes and segments of translocated chromosomes were rapidly delineated in the very complex karyotypes of such cells. Additional subregional probes were also used to further define aberrant chromosomes. Digital image analysis was used to quantitate the total complement of specific chromosomal DNAs in individual metaphase and interphase cells of each line. Under-representation of chromosome 21 and over-representation of chromosome 7 (specifically 7p) were observed. This is in agreement with previous observations by others using conventional cytogenetic banding techniques. Bigner, S.H. et al., *Cancer Genet. Cytogenet.*, 22:121-135 (1987); Shapiro, J.R., *Semin. Oncol.*, 13:4-15 (1986).

[0020] The two glioma cell lines used display several cytogenetic features common to many glioma cells. Thus, it is reasonable to expect that the CISS hybridization method can be used in a similar manner to specifically delineate other chromosomes and to detect those chromosomes in glial tumors. The two cell types analyzed are highly aneuploid (i.e., they have 100 chromosomes, rather than the normal 46). Therefore, it is reasonable to expect that the CISS hybridization method can be used in assessing any type of aneuploid (tumor) cell.

[0021] Thus, the CISS hybridization method can be used in assessing chromosomal aberrations associated with cancer, both in diagnosis of the disease and in monitoring its status (e.g., progression, regression or change with treatment) in patients. In this application, assessment of a single chromosome or of multiple chromosomes, and subregions thereof, can be carried out. Double hybridizations using two DNA probes, each bearing a different label can also be carried out. That is, biotinylated chromosome 7 library DNA inserts and a probe specific for alphoid repeats on chromosome 7 (pa7tl) which was modified with aminoacetylfluorene (AAF) were used to assess chromosome 7 content/characteristics in both metaphase spreads and interphase nuclei of the two types of tumor cells (TC 593, TC 620). After hybridization, biotinylated chromosome 7 inserts were detected using avidin-FITC and chromosome 7-specific alphoid AAF labeled sequences were detected with tetramethylrhodamine isothiocyanate (TRITC) conjugated second antibodies. Double CISS hybridization was used to detect translation between chromosome 8 and 14, Burkitt lymphoma cells, a high malignancy form of B lymphocyte tumors such were seen in both metaphase spreads and interphase cells.

[0022] This made it possible to detect similarities and differences in chromosome number 7 present in the two tumor cell types: only the four complete number 7 chromosomes found in TC 593 contained a detectable 7 centromeric signal; a smaller and metacentric number 7 chromosome lacked the 7 alphoid sequences and a small block of heterochromatin at 7q11 (indicating that it lacked a characteristic centromeric region). In contrast, all four chromosome number 7 of TC 620 were labeled with the 7 alphoid probe. Double CISS hybridization also made it possible to distinguish among number 7 chromosomes present in one cell type (TC 593) and to demonstrate similarity (at least as to the characteristics assessed) among number 7 chromosomes present in the other cell type (TC 620).

[0023] Double CISS hybridization was used to detect translocations between chromosome 8 and chromosome 14 in Burkitt's lymphoma cells; Burkitt's lymphoma is a highly malignant form of B lymphocyte tumors. Translocations were detected in both metaphase spreads and interphase cells.

[0024] It is possible, through the use of appropriately-selected probes and/or labels to increase the number of different chromosomes, as well as the number of subregions on some or all of those chromosomes, which can be analyzed simultaneously using multiple CISS hybridization. For example, it is possible to use more than one probe, each specific for a subregion of a target chromosome, to analyze several subregions on that single chromosome at one time. It is also possible to label each probe set (set of DNA or RNA fragments) with a distinct fluorochrome or different reporter molecule, which can be distinguished from one another, after probe-target chromosome hybridization has occurred, by known techniques (e.g., by using specific fluorescent or enzyme reagents).

[0025] Furthermore, a "combinatorial" variant of CISS hybridization can be used to enhance the number of chromosomes which can be assessed simultaneously. That is, it is possible to use a hybridization probe mixture made from a single set of probe sequences composed of two halves, each separately labeled with a different fluorochrome (e.g., fluorescein and rhodamine), which, upon hybridization, produce a third fluorescence "color" or signal optically distinguishable from each of the original individual fluorochromes. Pairing of two different fluorochromes in this manner makes it possible to identify three different chromosomes. For example, a probe set labeled only with fluorescein will yield one color upon hybridization; the same probe set labeled only with rhodamine will yield a second (different) color upon hybridization. When half of the probe set is labeled with one of the two, both sequence subsets can hybridize to target with equal probability and be perceived as a third (different) color (in a way not dissimilar to mixing paint). It is important here that two fluorochromes are not introduced into the same molecule, in order to minimize the possibility of E transfer (a well-known process where light emitted by one fluorochrome whose spectrum overlaps that of the other fluorochrome is absorbed by the second fluorochrome. The transferred electrons are emitted by second fluorochrome, which leads to quenching of the first fluorochrome). Pairwise combinations of three different fluorochromes selected for their spectral characteristics can be used singly and in pairwise combinations to produce in a similar manner. This can result in the production of six different fluorescent colors or signals (e.g., three pairs plus three single fluorochromes). Similar combinations of four different fluorochromes results in production of 10 different fluorescent colors or signals, of

five different fluorochromes results in production of 15 different colors or signals, etc. This principle of combinatorial fluorescence (combining two or more fluorochromes to label the same probe set) is applicable to metaphase and interphase chromosome analysis because each chromosome is a physically separate entity and is, thus, a distinct target. Composite probe labeling in which mixtures of three different fluorochromes are used provides even greater diversity of colors or signals useful in simultaneous multiparameter analysis.

[0026] Another approach to enhance the number of chromosomes which can be analyzed simultaneously involves a "time-reolved" method of fluorescence detection. In this instance, the DNA (or RNA) probes are labeled with chelating "cages" which bind specific lanthanides (e.g., Europium, turbium). Such metal chelates can be made to fluoresce. They exhibit excited state lifetimes that are much longer (micro to millisec) than those of most normal fluorochromes (whose half lives are in the nanosecond range). Both the wavelength and the fluorescence lifetime is influenced by the nature of the lanthanide metal ion employed. If a pulsed-gating system, which excites the sample with light for a few nanoseconds and then shuts off is used, it is possible to let short-lived fluorochromes decay to their ground state, open the detector system at a defined time after excitation, (i.e., 1-100 microseconds) and detect only long-lived fluorochrome. This method can be used to discriminate 2 fluorescent dyes which have identical spectra but different lifetimes, thus adding a time factor to fluorochrome discrimination.

[0027] Another approach to increase the number of different chromosomes that can be analyzed simultaneously is based on a detection system which distinguishes chromosomes in terms of the flexibility or rigidity of an attached fluorochrome. Here, two single stranded probe sets can be labeled with the same fluorochrome, in one probe set the fluorochrome is introduced into the body of DNA sequences which will form hybrid molecules with the target DNA of interest. In the second probe set, the fluorochrome is introduced into DNA sequences, that do not hybridize with the target DNA (e.g., by adding a 3'-tail of poly dA-fluorochrome with deoxynucleotide terminal transferase, ligation of fluorochrome-labeled heterologous DNA to the probe DNA or other conventional secondary labeling techniques known in the art). Fluorochromes within the body of the DNA which form probe-target chromosome hybrids will become immobilized and thus will be unable to rotate freely in solution. In contrast, fluorochromes in the single-strand DNA that is not involved in hybrid formation are not immobilized and can rotate much more freely in solution. By measuring the rate of fluorochrome rotational freedom, (i.e., by measuring how fast the fluorochromes become depolarized when illuminated with polarized light) one can discriminate the two sets of probes.

Use of CISS hybridization and regionally defined probe sets for rapid assessment of chromosome aberrations associated with genetic disorders and chromosomal damage

[0028] It has been demonstrated that the CISS hybridization method is useful for the rapid assessment of chromosome aberrations (such as numerical and structural aberrations of chromosome 21) associated with genetic disorders (e.g., in the case of chromosome 21, Down syndrome). DNA probe sets which specifically label the terminal band 21q22.3 or decorate the entire chromosome 21 aberrations in metaphase and interphase cells are described in Example 3. The cloned DNA fragments from the human chromosome 21 are useful to specifically label the cognate chromosomal region in metaphase spreads and interphase nuclei in a variety of cell types. That is, CISS hybridization using a chromosome 21 probe set was shown to be effective in labeling/identifying chromosome 21 DNA in lymphocytes, embryonic chorionic villi cells and a glioma tumor cell line (TC 620). Unique probe sets from band 21q22.3 were also used to detect chromosome 21 in solid tissue ("normal" human brain tissue). Thus, CISS hybridization and hybridization with pools of unique sequence probes clearly have potential as a diagnostic for Down syndrome and for other genetic diseases or other conditions associated with chromosomal aberrations.

[0029] Results demonstrate that a trisomic karyotype can be diagnosed easily in interphase cells because the majority of the nuclei (55-65%) exhibit three distinct foci of hybridization. In contrast, less than 0.2% of nuclei in lymphocytes with a disomic karyotype show three nuclear signals; interestingly, the percentage of such nuclei in normal CV cells was higher but still considerably less than 5%. In general, as few as 20-30 cells were sufficient to unambiguously distinguish between disomic and trisomic cell populations. However, in view of the uncertainty of the level of chromosome 21 mosaicism in clinical samples, the number of cells required to make an unambiguous diagnosis will likely be higher. Additional clinical correlations will be required to establish the absolute number. Nevertheless, this analytical approach could allow the diagnosis of Down syndrome without the need to culture cells or to obtain metaphase spreads. It would also decrease the time required to make the diagnosis, from the current 10-14 days to 1 day or less.

[0030] Although selected plasmid clones containing only unique human DNA sequences were used here, cosmid clones containing repetitive sequences can also be used to specifically label their cognate genomic region in metaphase and interphase cells by applying hybridization protocols like CISS hybridization that suppress the signal contribution of repetitive sequence elements. Therefore, single or nested sets of cosmids could be used as diagnostic tools for other genetic diseases in a fashion similar to that reported here. Trisomy of chromosomes 13, 18 and 21 and numerical changes in chromosomes X and Y together account for the vast majority of numerical chromosome abnormalities identified during prenatal karyotyping. With the continued development of multiple nonisotopic probe labeling and

detection systems it should be possible to visualize three or more chromosomes simultaneously following *in situ* hybridization. The variations, described in the previous section, of the CISS hybridization method which increase the number of chromosomes, and/or the number of chromosome regions which can be assessed simultaneously can also be used for detecting chromosomal aberrations associated with genetic disorders and chromosomal damage. Thus, the development of a rapid and automated screening test to detect the major trisomic disorders directly in interphase cells from amniotic fluid or chorionic villi cells is a viable future objective. The analysis of specific human chromosomes by *in situ* hybridization has already been used to complement conventional cytogenetic studies of highly aneuploid tumor lines (Example 2) and the extension to prenatal diagnostic applications seem warranted.

[0031] The analysis of karyotypes with translocations of chromosome 21 shows the usefulness of a regional probe set to rapidly identify and characterize even small translocations by unambiguous signals on metaphase chromosomes, thus circumventing an extensive analysis by high-resolution banding. In contrast, the library insert probe is more suitable for defining the relative amount of chromosome 21 DNA that has been translocated. By analyzing interphase nuclei, one can also determine if a balanced or unbalanced number of chromosomal regions exists. However, the detection of a translocated chromosome directly in nuclei would require double-labeling techniques to identify the recipient chromosome to which the chromosome 21 material was translocated. With prior knowledge of the chromosome in question, such translocation events could be assessed by measuring the juxtaposition of the nuclear signals. Rappold, G.A. et al., *Hum. Genet.*, 67:317-325 (1984).

[0032] A cosmid clone spanning the entire muscular dystrophy (MD) locus on chromosome X has been used to identify translocation between chromosome X and chromosome 4.

[0033] Probes containing 6 kb of sequence were localized in both metaphase spreads and interphase cells with high efficiency. This detection sensitivity with nonisotopic reagents is similar to that achieved in other recent reports. The combination of nonisotopic *in situ* hybridization with DAPI or BrdUrd banding or total chromosome decoration with library DNA probes thus provides a simple and general approach for gene mapping. Combinatorial fluorescent technology will also make it possible to examine several chromosomal regions simultaneously, thus permitting genetic linkage analysis by *in situ* hybridization. It also should facilitate the use of small DNA probes to rapidly pinpoint the breakpoints on translocation chromosomes, which could further aid in defining the genomic segments critical for Down syndrome.

Identifying and Isolating Chromosome-Specific Sequences Using CISS Hybridization

[0034] The CISS hybridization method of the present invention can also be used to identify chromosome-specific sequences and, subsequently, to separate them from repetitive sequences, using known techniques. Such chromosome-specific sequences, separate from the non-specific or repetitive sequences, and labeled, can be used in hybridization assays carried out, for example, in a diagnostic context, to identify, detect, and/or quantitate a chromosome or chromosome region of interest (e.g., one which is associated with a genetic disorder or causes an infectious disease). Combination of a sample to be assayed for a selected target nucleic acid sequence or sequences and appropriately-selected, labeled chromosome-specific sequences separated from repetitive sequences (e.g., sequences specific for sequences on the chromosome(s), generally referred to as target nucleic acid sequences, which are to be detected and/or quantitated in the sample under appropriate conditions results in hybridization with complementary sequences present in the sample. Hybridization will not occur, of course, if complementary sequences are not present in the sample.

[0035] Such separated chromosome-specific nucleic acid sequences can be incorporated into a kit to be used for identification, detection and/or quantitation of chromosomes or chromosome regions of interest, using standard hybridization techniques. For example, labeled nucleic acid sequences which are chromosome 21 specific (or specific to a portion of chromosome 21), identified by CISS hybridization, and separated from repetitive sequences present on chromosome 21, can be included in a kit, along with other reagents such as buffers, competitor DNA, carrier DNA and substances needed for detection of labeled chromosome 21-derived nucleic acid sequences hybridized to chromosome 21 sequences present in a sample. Such kits clearly can be produced to include chromosome-derived nucleic acid sequences from one or more chromosome(s) of interest. Competitor DNA, carrier DNA and substances useful for detecting hybridized sequences will be as described above.

EXAMPLE 1 Cyto-Specific Staining of Individual Human Chromosomes Using Genomic DNA Libraries in CISS Hybridization

DNA libraries

[0036] The following human chromosome genomic libraries were obtained from the American Type Culture Collection: LA01NS01 (chromosome 1), LL04NS01 (chromosome 4), LA07Ns01 (chromosome 7), LL08NS02 (chromosome 8), LA13NS03 (chromosome 13), LL14NS01 (chromosome 14), LL19NS01 (chromosome 18), LL20NS01 (chromo-

some 20), LL21NS02 (chromosome 21), LA22NS03 (chromosome 22), LA0XNL01 (chromosome X). Amplification of these phage libraries on agar plates (using LE 392 cells as the bacterial host), purification of the λ phages and extraction of phage-DNA pools were carried out according to standard protocols. Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NJ (1982).

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Preparation of metaphase spreads and fibroblast cells

[0037] Phytohemagglutinin-stimulated lymphocytes from a normal adult male (46, XY) were cultured in McCoy's 5A medium (GIBCO), arrested with Colcemid, treated with a hypotonic solution of 0.075 M KCl, fixed in acetic acid-methanol and metaphase spreads made by standard procedures. Low-passage normal human foreskin fibroblasts (46, XY) 10 were grown on microscope slides, fixed with paraformaldehyde, and permeabilized as described for study of preparations with a more intact three-dimensional structure. Manuelidis, L., Ann. NY Acad. Sci., 450:205-221 (1985).

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Preparation of DNAs for in situ hybridization

[0038] Insert DNA probes. Genomic DNA fragments from the chromosomal DNA libraries were separated as a pool 15 from the Charon 21A vector arms by digestion with the appropriate restriction enzyme [EcoRI (LA libraries) or Hind III (LL libraries)], followed by preparative electrophoresis in 0.6% agarose gel. The insert fragments were isolated from gel slices by electroelution into an Elutrap (Schleicher and Schuell) and further purified by Elutip-d column chromatography 20 (Schleicher and Schuell). The DNA was then extracted with phenol/chloroform (1:1) and ethanol precipitated. This pool of DNA fragments was labeled either by nick translation using Bio-11-dUTP or by random primer extension with the multiprime DNA labeling system (Amersham) substituting dTTP with 0.5 mM Bio-11-dUTP. Langer, P.R. et al., Proc. Natl. Acad. Sci. USA, 78:6633-6637 (1981) and Brigati, D.J. et al., Virology, 126:32-50 (1983). Alternatively, the DNA of the 25 chromosome-specific libraries was biotin-labeled directly (without separation of the vector arms) by nick translation.

[0039] Probe size. To facilitate probe penetration and to optimize reannealing hybridization, labeled DNA fragments 30 smaller than 500 nucleotides are used; the majority of the probes are generally 150 to 250 nucleotides in length. DNase concentrations were empirically established in nick-translation reactions to yield fragments in the desired size range and this was verified by agarose gel electrophoresis. Random primer extensions were also carried out under conditions which yielded a comparable DNA size distribution.

[0040] Competitor DNA. Human genomic DNA (from placenta or white blood cells), prepared as described, as well 35 salmon testis genomic DNA (Sigma) were digested with DNase to obtain fragments with the same size distribution as the probe DNA, then extracted with phenol/chloroform and ethanol precipitated. Davis, L.G. et al., "Basic methods in molecular biology", Elsevier, New York Amsterdam (1986). These competitor DNAs were used in varying ratios with probe sequences, as described with reference to Figure 2.

[0041] Preannealing and hybridization. Under standard conditions, from 5 μ g/ml to 30 μ g/ml of biotin-labeled DNA, 40 representing library insert fragments, and varying amounts of competitor DNAs were combined, ethanol-precipitated and resuspended in formamide. The probe concentration was adjusted to reflect the relative DNA content of each chromosome target. For example, chromosome 1 contains approximately 5.3 times as much DNA as chromosome 21; thus the probe concentrations used were 30 μ g/ml and 5 μ g/ml, respectively. Mendelsohn, M.L. et al., Science, 179:1126-1129 (1973). When total library DNA was used as the probe mixture instead of purified DNA inserts, 10 times as much 45 labeled DNA was added to compensate for the large amount of vector sequences. In the case of the X-chromosome library, LA0XNL01, only twice as much labeled library DNA was used, since the human DNA inserts constitute almost half of the total DNA. For comparative purposes, the concentration of human competitor DNA in the hybridization mixture was varied from 1 to 1.0 mg/ml and salmon testis DNA was added as required to result in a final DNA concentration 50 of 1.0 mg/ml in 50% formamide, 1 X SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and 10% dextran sulfate. These solutions were heated at 75°C for 5 min. to denature the DNA and then incubated at 37°C for various times to promote partial reannealing. The preannealing step was done in an Eppendorf tube just prior to applying the hybridization mixture to the specimen. Nuclei and chromosome spreads on glass slides were incubated in 70% formamide, 2 X SSC] at 70°C for 2 min. to denature chromosomal DNA and then dehydrated in a series of ice-cold ethanal (70%, 90% and 100%, each for 3 min.). After application of the preannealed probe mixture (2.5 μ l/cm²) to slides pre-warmed to 42°C, a coverslip was added and sealed with rubber cement. The samples were then immediately incubated 55 at 37°C in a moist chamber for 10-20 h.

[0042] In those cases where paraformaldehyde fixation was used to more optimally preserve the 3-D structure of the specimen, the slides were equilibrated in 50% formamide, 1 X SSC (2 X 5 min.), excess fluid was removed without permitting the sample to dry, the probe mixture was added (5 μ m/cm²), and a coverslip mounted and sealed with rubber cement. Manuelidis, L., Ann. NY Acad. Sci., 450:205-221 (1985). Denaturation of both probe and cellular DNA was done at 75°C for 5 min. before hybridization was allowed to proceed overnight at 37°C.

Detection

[0043] After hybridization, the slides were washed in 50% formamide, 2 X SSC (3 X 5 min., 42°C) followed by washes in 0.1 X SSC (3 X 5 min., 60°C). Thereafter the slides were incubated with 3% bovine serum albumin (BSA), 4 x SSC for approximately 30 minutes at 37°C. Detection of the biotinylated probe was achieved using either fluorescein-labeled avidin or avidin-alkaline phosphatase complexes. All detection reagents were made up in 4 X SSC, 0.1% Tween 20, 1% BSA and all washes were carried out in 4 X SSC, 0.1% Tween 20 (3 X 3 min., 42°C). For fluorochrome detection, slides were incubated with 5 µg/ml fluorescein isothiocyanate (FITC)-conjugated avidin DCS (Vector Laboratories) at 37°C for 30 min., followed by washes. In rare cases, the FITC signal was amplified by incubation with 5 µg/ml biotin-conjugated goat anti-avidin D antibodies (Vector Laboratories) at 37°C for 30 min., followed by washing, a second incubation with 5 µg/ml FITC-conjugated avidin (37°C, 30 min.) and a final wash. Pinkel, D. et al., *Proc. Natl. Acad. Sci. USA*, 83:2934-2938 (1986). For detection by enzyme activity, samples were incubated with 2.5 µg/ml streptavidin, washed, incubated with 2 µg/ml biotin-conjugated alkaline phosphatase (Vector Laboratories), washed again and pre-equilibrated in AP-buffer 9.5 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 2 x 5 min. at room temperature.

The enzyme reaction was carried out in AP buffer 9.5 containing 330 µg/ml of nitroblue tetrazolium (NBT) and 165 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at 37°C for 0.5-1 hour and stopped by incubation in 2 X SSC. All preparations were counterstained with 200 ng/ml 4,6-diamidino-2-phenylindolehydrochloride (DAPI), 2 X SSC for 5 min. at room temperature and mounted in 20 mM Tris-HCl, pH 8.0, 90% glycerol containing 2.3% of the DAPCO antifade, 1,4 diazabicyclo-2(2,2,2)octane. Johnson G.D. et al., *J. Immunol. Methods*, 55:231-242 (1982).

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Densitometry

[0044] A graphics workstation (VAX station II/GPX, Digital Equipment Corporation) with a frame grabber (ITEX FG-101, Imaging Technology) and a Dage-MTI-65 video camera with a Zeiss S-Planar 60 mm lens were used as described in Manuelidis, L. and J. Borden, *Chromosoma*, 96:397-610 (1988). Images were digitized directly from the negatives and stored on disk. Background was removed and polygonal regions around each chromosome were defined. Threshold density levels were used to outline chromosome regions within the defined polygonal areas. Means density levels within these outlined chromosome regions, R, were determined by the total signal $\int I(x,Y)dR/\text{area } R$, where $I(x,y)$ is the pixel intensity (0-225) at each point within the region R. The threshold background intensity was subtracted from the mean regional density, both for labeled chromosome 7 and for background chromosomes. The signal to noise ratio was calculated as mean chromosome 7 signal/mean background chromosome signal.

[0045] The following is a description of the results of the work described above, which clearly demonstrate specific labeling of the individual chromosome indicated. The first sections describe use of chromosome library inserts labeled with biotin and the second describes use of DNA insert fragments.

[0046] Figure 2 shows suppression of signals from cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations, as described below.

[0047] Figure 2A shows chromosome 7 library inserts labeled with biotin and hybridized to metaphase spreads from normal human lymphocytes without human competitor DNA. Prominent labeling of the two no. 7 chromosomes is observed; additionally, a distinct band-like patterns of hybridization is seen on most of the other chromosomes, and two E-group chromosomes are especially brightly stained. This general chromosomal banding pattern resembles R-banding, and suggests that a significant portion of the background cross-hybridization signal originates from Alu repetitive sequences. Previous studies have shown that Alu sequences delineate an R-banding pattern, while Giemsa positive-banding profiles are highlighted by KpnI interspersed repeats. Manuelidis, L. and C.D. Ward, *Chromosoma*, 91:28-38 (1984).

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Establishment of experimental procedure to eliminate the hybridization signal from repetitive elements

[0048] A series of pilot studies were therefore undertaken to establish experimental parameters to eliminate the hybridization signal from such repetitive elements. The kinetics of nucleic acid reassociation in solution are dependent on the total concentration of nucleic acid (Co, in moles of nucleotides per liter) and the time of renaturation (t, in seconds). When reassociation conditions are standardized for temperature (taking into account the formamide concentration), cation concentration and buffer system, the reassociation kinetics are comparable with respect to Cot values. Under defined conditions, the fast reassociating fraction of mammalian genomes containing the highly repetitive DNA is completely reannealed at Cot values between 1×10^{-1} and 5×10^{-1} ; the intermediate fraction containing the middle repetitive DNA is completely renatured at a Cot value of 1×10^2 . Britten, R.J. and D.E. Kohne, *Science*, 161:529-540 (1968). Thus at a human DNA concentration of 1.0 mg/ml (corresponding to 3×10^{-3} moles of nucleotide per liter), the fast fraction would be renatured in approximately 10s, whereas the middle repetitive DNA would need more than 9 h to reach complete reannealing. Since the fast fraction of reassociating DNA containing most or all of the ubiquitous repet-

itive DNA causing cross-hybridization signals, a total DNA concentration of 1.0 mg/ml was used and partial reannealing of the probe mixture was allowed prior to application to specimens. The optimal renaturation time was determined empirically (see below). This was important because the *in situ* hybridization conditions deviate from the standard conditions under which reassociation kinetics are determined (e.g., hybridization in 50% formamide at 37°C corresponds to 0% formamide at about 70°C; dextran sulfate also increases the reassociation time significantly). Furthermore, it was unclear to what degree the middle repetitive DNA contributed to the non-specific signal and therefore should also be prevented from hybridization by a preannealing procedure.

[0049] The stringency for the reannealing and *in situ* hybridization experiments was determined in 50% formamide at 37°C (adapted from standard *in situ* hybridization protocols) and 1 X SSC [this cation concentration of 0.165 M comes close to the concentration used in kinetics the study of Britten and Kohne. Britten, R.J. and D.E. Kohne, *Science*, 161:529-540 (1968)]. Competitor human DNA was added in the reassociation procedure to obtain the desired final high DNA concentration and to maintain a high level of repetition of the DNA sequences that should preanneal. While total human genomic DNA represents all the highly repetitive DNA to be removed by pre-annealing, it also contains sequences of the target chromosome. Thus, the addition of excessive amounts of human DNA would be expected to diminish the chromosome-specific signal. Therefore, the optimal concentration of total human DNA to use as the competitor was first determined. To keep the total DNA concentration constant at 1.0 mg/ml, genomic salmon DNA was added as needed. Salmon DNA shares certain repetitive DNA elements, such as poly dCdA in common with human DNA, but lacks others, most notably the Alu- and KpnI repeats. Hamada, H. et al., *Proc. Natl. Acad. Sci. USA*, 79:6465-6469 (1982). This results in a lower frequency of the latter sequences with increasing amounts of salmon DNA in the reassociation reaction.

[0050] Figure 2 shows typical experimental results obtained when 20 µg/ml of the chromosome 7 probe set was denatured together with 50 µg/ml (B), 100 µg/ml (C), 200 µg/ml (D) or 1000 µg/ml (E) of DNase-digested human genomic DNA which was preannealed for 20 min. Hybridization and detection using avidin-FITC were carried out as described above. From each preparation ten black and white pictures were taken under standardized photographic conditions for densitometric studies (see below). In the absence of human genomic competitor (A) the signal showed little chromosomal specificity. However, with 50 and 100 µg/ml of human competitor DNA, an increase of label specificity is readily apparent (Figure 2B,C). Specific staining of chromosome 7 was achieved with a peak of signal intensity using 100 and 200 µg/ml of human competitor DNA (Figure 2C, D). Higher concentrations of human DNA caused an apparent decrease of signal intensity, especially at 1000 µg/ml human DNA (Figures 2E). However, the signal obtained under these latter conditions is still reasonably bright to the observer, but requires a different exposure for optimal illustration (not shown).

[0051] A computer-assisted method of quantitative densitometry (see above) was used to establish the overall level of labeling specificity. The ratio of fluorescence signal from the target chromosomes of interest to the background fluorescence noise emanating from non-target chromosomes was determined from images digitized from multiple photographic negatives of a DNA titration experiment, as illustrated in Figure 2. The signal-to-noise ratio obtained with each concentration of human competitor DNA is given in Table 1.

Table 1

| Densitometric analysis of the suppression of cross hybridization signals by concentrations of human competitor DNA | | | | | | | |
|--|------------------|----------------------------|--------------------|-------|--|----|-----------|
| | DNA conc. µg/ml) | Human competitor DNA conc. | Signal | Noise | Signal-to-noise ratio Confidence interval ^c (99%) | | |
| 45 | 0 | | Pixel ^a | n | Pixel ^b | n | 1.31±0.04 |
| | 50 | | 71.48 | 8 | 54.66 | 26 | 1.99±0.07 |
| | 100 | | 74.50 | 8 | 37.43 | 28 | 8.11±0.35 |
| | 200 | | 162.64 | 8 | 20.06 | 23 | 7.18±0.37 |
| | 500 | | 147.35 | 8 | 20.53 | 26 | 4.82±0.28 |
| | 1000 | | 89.78 | 8 | 18.63 | 21 | 3.09±0.12 |
| | | | 94.37 | 8 | 30.51 | 17 | |

^a Mean value of pixel intensity of target chromosome

^b Mean value of pixel intensity of non-target chromosomes (from the same metaphase spreads)

^c The confidence interval was calculated using Fieller's theorem (Finney, D.J., Statistical methods in biological assay, 2nd edn., Hafner Press, N.Y., 1971)

n Number of chromosomes from which the mean was determined

[0052] Optimal reannealing conditions for suppression of nonspecific signal (using 20 µg/ml of chromosome 7 probe and 100-200 µg/ml human genomic (DNA), gave a signal-to-noise ratio of ca. 8:1. Additional attempts to improve the signal to noise ratio by increasing hybridization stringencies (e.g., 60% formamide or 0.2 x SSC) gave no apparent improvement and led to an overall decrease in signal intensity.

5 [0053] Since about 100-200 µg/ml of human competitor DNA was shown to give the optimal specificity, 200 µg/ml was used for another analysis of signal specificity with respect to the renaturation time (see above). After 0, 2, 5, 10, 20, 40 and 50 min. of preannealing, aliquots were taken and used for *in situ* hybridization experiments as before. As indicated in Figure 3, specific labeling was obtained for all preannealing times. A small improvement of the signal is seen with increasing renaturation times from 0 to 20 min. Longer renaturation times up to 60 min. (not shown) gave no
10 significant improvement in signal strength or chromosome specificity. The subjective impression of a signal improvement with 20 min. of preannealing (Figure 3D) could not be confirmed by a densitometric analysis, carried out as described above, since no significant differences in the signal-to-noise ratio of the different preannealing times were observed (data not shown). Therefore, the standard renaturation time in all subsequent experiments was 10-20 min.
15 Since a signal is clearly visible at renaturation time 0, the few seconds necessary for transferring the probe mixture to the microscope slide appear to be sufficient to effectively preanneal many of the sequences that cause nonspecific labeling by cross-hybridization. Furthermore, the large excess of single-stranded competitor DNA may efficiently compete with the biotinylated probe sequences for ubiquitous chromosomal target sites during the hybridization reactions. These results demonstrate that the majority of highly repetitive DNA sequences can be sufficiently suppressed to achieve chromosome-specific labeling by *in situ* hybridization.

20 [0054] In certain cases the signal distribution over the entire chromosome shows some variability from experiment to experiment. When the overall signal is decreased, some chromosomal subregions show a brighter staining; these signal hotspots generally constitute chromosomal sites that contain known chromosome-specific repetitive sequences. In the experiments shown in Figures 2 and 3, predominant staining of the centromeric region of chromosome 7 is seen, which corresponds to the chromosome-specific signal of an alploid repetitive DNA. Waye, J.S. et al., Mol. Cell Biol.,
25 7:349-356 (1987) and see Example 2. Apparently, the abundance of these repeated sequences is sufficiently low to prevent their suppression under the conditions used here. The unequal signal distribution can be overcome by amplifying the overall signal using an antibody sandwich technique as described above. Furthermore, a predominant staining of the region 1q12 that corresponds to the chromosomal site of satellite III DNA was frequently observed in labeling chromosome 1. Cooke, H.J. and J. Hindley, Nucleic Acids Res., 6:3177-3197 (1979) and Gosden, J.R. et al., Cytogenet.
30 Cell Genet., 29:32-39 (1981) and see Example 2. An example of the balanced signal distribution seen after such an amplification step is shown in Figure 4A.

[0055] Several commercially available DNA libraries, each representing a single human chromosome, were tested for their ability to specifically label the chromosome they represented, under the standardized reannealing conditions described above and with the probe concentrations adjusted for chromosome size, as described above. Some examples, for chromosomes 1, 4, 7, 13, 18 and 20, as shown in Figure 4, clearly demonstrate that specific labeling can be achieved with most chromosome libraries. Table 2 lists the libraries tested with their relative scores of labeling specificity. All scores are positive because the chromosome of interest was always decorated. The highest score (4+) is used when no significant cross-hybridization to other chromosomes was observed and the scores decrease (3+ to 1+) with an increasing amount of cross-hybridizing sequences.

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Table 2

| Relative quality of specific chromosome labeling <i>in situ</i> using preannealed biotinylated library DNA inserts | | |
|--|---------------------------------|--|
| Chromosome | Library used (ATCC designation) | Relative specificity of <i>in situ</i> hybridization signal ^a |
| 1 | LA01NS01 | 3+ |
| 4 | LL04NS01 | 4+ |
| 7 | LA07NS01 | 4+ |
| 8 | LL08NS02 | 4+ |
| 13 | LA13NS03 | 1+ |
| 14 | LL14NS01 | 2+ |
| 18 | LL18NS01 | 4+ |
| 20 | LL20NS01 | 4+ |
| 21 | LL21NS02 | 3+ |
| 22 | LA22NS03 | 3+ ^b |
| X | LA0XNL01 | 4+ |

^a See the text for score definition

^b Under standard preannealing conditions the chromosome 22 library gave a score of +1; a value of +3 was achieved only with a human competitor DNA concentration \geq 700 μ g/ml (total DNA concentration 1.0 mg/ml).

[0056] All attempts to reduce the additional signals on other chromosomes by varying the experimental conditions failed except in experiments with chromosome 22; in this case higher concentrations of human competitor DNA (700 μ g/ml) resulted in a significant improvement of signal specificity. The library exhibiting the lowest chromosome specificity was the chromosome 13 library (Figure 4E). Multiple minor binding sites on other chromosomes, as well as an exceptionally bright staining of Yq12 were observed; the signal on the Y chromosome was visible using either female or male human DNA as the competitor. None of the experimental parameters tested improved on the overall specificity of this library.

[0057] Remarkably, a weak signal or even absence of signal can be observed at the centromeric region of some chromosomes (see chromosomes 4 and 18, Figure 4C,D). In contrast to chromosomes 1 and 7, which contain chromosome-specific repetitive elements, the centromere regions of chromosomes 4 and 18 apparently contain repetitive sequences, most likely alphoid satellite DNAs, which are very abundant and thus are suppressed by the reannealing technique. However, these chromosomal regions are very small and the effect can only be observed when the corresponding chromosomes are fairly elongated.

[0058] Biotinylated total library DNA (containing the phage vector sequences) was also used as probes, in concentrations adjusted to the amount of human DNA inserts. (see above). One example is shown in Figure 4F with the chromosome 20 library. Although good staining of the chromosome of interest generally was achieved, significant nonspecific background on the entire slide was a common problem. Similar results were obtained with plasmid libraries containing human DNA subcloned from the lambda phage libraries. In contrast, there was no background problem with the total chromosome library LA0XNL01, which contains a significantly smaller proportion of vector sequences in the probe mixture since the size of the human DNA inserts is much larger.

[0059] The suppression of repetitive sequences by this reannealing technique also permits the use of flow-sorted chromosome libraries to detect chromosomal domains within interphase nuclei. Typical examples of results obtained after hybridization of chromosome 1, chromosome 7 and chromosome 18 probe sets to normal human lymphocytes after acetic acid-methanol fixation are shown in Figure 5. Discrete focal domains of hybridization signal are seen with all libraries that had scores of 2+ or more (see Table 2).

[0060] Most nuclei ($n \geq 100$ per estimate) exhibited two domains (60%-70%); however, a significant number showed only a single domain (20%-30%) or no hybridization signal at all (5%-10%). Accordingly, ca. 95% of male nuclei exhibited one and ca. 5% showed no hybridization signal when the X chromosome library DNA was used as probe. Notably,

no nuclei with three domains were found with any of the chromosomal probe sets tested. In contrast, all metaphase spreads showed the decoration of both chromosome homologs without exception. This interphase variability may reflect, in part, the close juxtaposition of two individual domains in some cells, or the inability to resolve domains that actually occupy different areas within the nuclear volume but are unresolved when examined by two-dimensional imaging methods (see Fig. 5D; for discussion see also Cremer et al., *Exp. Cell Res.*, 176:199-220 (1988)). The small number of nuclei exhibiting no hybridization signal may be a reflection of suboptimal hybridization conditions. It is of interest to note that the size of the intranuclear domains correlates reasonably well with the relative size of the cognate metaphase chromosome. These observations provide a definitive proof that the DNA of individual chromosomes exhibits a clear territorial organization in the interphase nucleus of a normal human cell.

[0061] Acetic acid-methanol fixed nuclear spreads, such as those shown in Fig. 5, clearly retain the territorial organization for each of the chromosomes examined; however, the nuclear structure is not optimally preserved. Additional studies with specimens that possess better preservation of 3-D structure using paraformaldehyde fixed human diploid fibroblasts and a laser-scanning confocal fluorescence microscope assembly for 3-D image reconstruction have been done. The cells were fixed and permeabilized as described by Manuelidis and hybridized with chromosome library probes as outlined above. Manuelidis, L., *Ann. NY Acad. Sci.*, 450:205-221 (1985). The probe-competitor DNA mixture was applied directly to the slide and denatured at the same time as the cellular DNA. Results showed the arrangement of the chromosome 7 domains in the nucleus and the frequently observed helical structure of labeled chromatin within chromosome domains. The degree to which this helicity reflects true domain substructure or is an artifact reflecting preparation and fixation procedures is currently being investigated. Nevertheless, this preliminary observations establishes the feasibility of using chromosome specific probes to analyze the topography of chromosomal domains in the interphase cells.

EXAMPLE 2 Detection of Chromosome Aberrations in Tumor Cells by CISS Hybridization Using Chromosome-Specific Library Probes

25 Cells

[0062] TC 593 is a pseudotetraploid cell line (modal chromosome number, 83) established from a human glioblastoma; it grows in a flat, spreading fashion and contains many process. TC 620 is pseudotriploid with a modal chromosome number of 64 and was established from a human oligodendrogloma; it grows in an epithelial fashion. Both cell lines have been described in detail. Manuelidis, L. and E.E. Manuelidis, In: *Progress in Neuropathology*, Vol. 4, 235-266, Raven Press, N.Y. (1979). The present experiments made use of subclones C2B (TC 593) and C2B (TC 620) at approximately 180 passages after repeated subcloning from a single cell of the original tumor line cultured as previously described by Manuelidis and Manuelidis (see reference above). Standard hypotonic treatment and acid/methanol fixation of the cells were employed. Cremer et al., *Exp. Cell. Res.*, 176:199-220 (1988).

DNA Probes and Libraries

[0063] Phage DNA libraries from sorted human chromosomes were obtained from the American Type Culture Collection: LA01NS01 (chromosome 1), LL04NS01 (chromosome 4), LA07NS01 (chromosome 7), LL18NS01 (chromosome 18) and LA22NS03 (chromosome 22). Amplification of these libraries, isolation of human DNA inserts and biotinylation were carried out as described in Example 1. A probe specific for alphoid repeats on chromosome 7 (pa7tl) was the gift of H. Willard and specifically decorates pericentromeric heterochromatin of chromosome 7 under high stringency conditions (60% formamide). Waye et al., *Mol. Cell. Biol.*, 7:349-356 (1987); Cremer et al., *Exp. Cell. Res.*, 176:199-220 (1988). Some DNA probes were modified with aminoacetylfluorene (AAF); and detected as described by Cremer et al. for double labeling experiments. Landegent et al., *Exp. Cell Res.*, 153:61-72 (1984); Cremer, R. et al., *Exp. Cell Res.*, 176:199-220 (1988).

In Situ Hybridization and Detection of Hybridized Probes

[0064] CISS hybridization with biotinylated library DNA inserts and detection of hybrid molecules was generally carried out using standard conditions, as described in detail in Example 1. In double CISS hybridizations using biotinylated chromosome 7 library DNA inserts and the AAF-modified 7 alphoid probe, the latter probe was heat denatured separately and only added to the hybridization mixture at the end of the reannealing step at a final concentration of 10 µg/ml (see Example 1).

Digital Image Analysis of Specifically Decorated Metaphase and Interphase Chromosome

[0065] A VAX station II/GPX graphics workstation (Digital Equipment Corporation) with an ITEX FG 100-Q frame grabber (Imaging Technology) were used as previously described together with a Zeiss S-Planar 60 mm lens and a 5 Dage-MTI 65 video camera. Manuelidis, L. and J. Borden, *Chromosoma*, 96:397-410 (1988). Images were digitized from negatives of metaphase spreads and interphase nuclei; the background was removed and polygonal regions were defined to specifically decorated metaphase chromosomes or interphase domains (see Example 1). A scan line algorithm was used to calculate histograms within the polygonal regions. Since the value of the histograms $H(i)$ of a particular intensity (range 0-255) within the defined regions is the number of pixels at that intensity i , the area within the region 10 falling within an intensity range i_0-i_1 is the integral of the histogram from i_0-i_1 . Similarly, the 2-D integral in the region defined by the intensity range i_0-i_1 equals $\Sigma H(i).i.i_0$ was chosen for each hybridization, in order to properly outline the decorated chromatin and distinguish this area from background regions. i_1 was set to the maximum value 255 in order 15 to capture the entire intensity range above the threshold.

[0066] Measurements of total signal intensity versus area were designed as a control for the potential presence of 15 variable chromosome domain extension within interphase nuclei. In interphase, a more extended chromosome domain might be expected to have a greater area (or volume) yet a lower fluorescence signal intensity per unit area. If a constant amount of hybridized DNA corresponds to a constant total fluorescence, the total signal intensity is a measure of labeled DNA content. It is also possible to measure 3-D hybridized volumes within nuclei and 3-D integrated total 20 hybridized signals. Manuelidis, L. and J. Borden, *Chromosoma*, 96:397-410 (1988). The background, b , was subtracted from the discrete 2-D integral $\iint I(x,y)dA$ within a labeled region R , to yield the total signal:

$$\text{Sig}_t = \iint I(x,y)dA - b \iint dA,$$

where dA is a single pixel. Similarly, the mean intensity within the region is calculated as 2-D integral/area or 25 $\iint I(x,y)dA / \iint dA$.

[0067] The following is a description of the results, with reference to the appropriate figures, of the work described above. They clearly document structural and quantitative changes in the human glioma lines, including loss and gain of entire individual chromosomes and of chromosomal subregions. They also show that it has been possible to characterize both minor and predominant karyotypic features in each cell line. All chromosomes tested to date (i.e., 1, 4, 7, 18 30 and 22) clearly highlighted numerical and/or structural aberrations, some of which were subtle.

[0068] Detection of numerical and structural chromosome aberrations in metaphase spreads.

[0069] Figures 6-8 and 12 show typical metaphase spreads from the malignant glioma cell lines TC 620 and 593 after CISS hybridization with biotinylated DNA inserts from each of the human chromosomes 1, 4, 7, 18 and 22. Hybridized inserts were detected with avidin fluorescein isothiocyanate conjugates (FITC) and cells were counterstained with 35 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Chromosomes designated as "complete" had an apparently normal size, centromere index and DAPI staining pattern. Despite this designation, these complete chromosomes may contain fine structural aberrations only detectable by additional investigations (see below). Apparently complete chromosomes 1, 4, 7, 18 and 22 were observed in both TC 620 and TC 593. Additionally, other homologs of these chromosomes showed significant rearrangements and abnormalities, including translocations and deletions. The predominant 40 numerical and structural aberrations delineated in each of these cell lines are described below. A minimum of 25 good metaphase spreads were evaluated for each glioma line and for each chromosome. These data are summarized in Fig. 9.

Chromosome 1

[0070] In TC 620, the oligodendrogloma line, chromosome 1 inserts decorated two apparently complete 1 chromosomes and two marker translocation chromosomes (Figs. 6A, 6B, 9). One marker was metacentric and contained an entirely decorated 1q arm, but its p arm was from another chromosome (of unknown origin). The other marker chromosome was submetacentric and showed a small segment from another chromosome attached to the 1p arm. In both 50 marker chromosomes breakpoints were localized close to the centromere in 1p11 or 1q11. The identification of the 1p segment was established by DAPI banding (Fig. 6B), by 5'-bromo-2'-deoxyuridine (BrdU) banding, and by hybridization with a 1p36.3 probe (data not shown); the 1p36.3 probe additionally revealed deletion of this subregion in one of the apparently complete 1 chromosomes. The overall picture was of a nearly trisomic representation of chromosome 1, with a common breakpoint, and subsequent translocation.

[0071] In TC 593, the glioblastoma line, an even more complex pattern of numerical and structural chromosome 1 aberrations was observed. In a sample of 50 metaphase spreads, the majority (52%) showed six aberrant chromosomes that were decorated; 14% of the metaphases showed five aberrant chromosomes, and 34% showed higher numbers of chromosomes 1 segments (up to 14). Figures 6C, D and 9 show the most typical, predominant karyotype

and demonstrates the rapid definition of chromosome 1 abnormalities in this cell line. Aberrations included three acrocentric chromosomes with a consistent breakpoint in 1p1, chromosomes with a deletion of the distal part of 1q, a submetacentric translocation chromosome with a loss of the complete 1q, and an iso(1p) marker chromosome (see Fig. 9).

5 Chromosome 4

- [0072] In TC 620, chromosome 4-specific inserts decorated one apparently complete chromosome 4, and three additional chromosomes with segments containing chromosomes 4 DNA (Figs. 7F, 9). These latter segments on translocation chromosomes would have been difficult to rapidly and unambiguously define with banding procedures alone.
- 10 The smallest of the translocated chromosome 4 segments formed part of an approximately metacentric chromosome. The two larger segments were found on submetacentric chromosomes of different overall size. In the smaller chromosome, the short arm and part of the long arm of 4 were present with an apparent breakpoint at 4q2, i.e., 4pter-4q2. In the larger submetacentric chromosome, a region that may represent the rest of 4 (4q2-qter) appears. Thus the predominant karyotype of TC 620 showed only slightly more than two equivalents of chromosomes 4 (see also the area measurements described below). The non-4 regions have not been further defined.
- 15 [0073] In TC 593, there were generally only two chromosomes decorated by chromosomes 4 DNA inserts, and both of these were compatible with normal 4 chromosomes. Approximately 30% of the metaphase spreads in TC 593 showed an additional submetacentric chromosome with chromosome 4 material (Fig. 7E). Thus, although both 4 chromosomes were apparently normal, there was a significant under-representation of this chromosome in this pseudodotetraploid line (Fig. 9).

Chromosome 7

- 20 [0074] Three complete 7 chromosomes, and one smaller metacentric chromosome containing translocated 7 material were typically found in TC 620 metaphase spreads (Figs. 8A, 9). The translocated chromosome 7 material included the short arm of chromosome 7 (as shown by DAPI banding; cf. Fig. 8B) and the pericentromeric heterochromatin with the breakpoint in 7q1 (see also below).
- 25 [0075] In TC 593, five chromosomes entirely decorated by chromosome 7 insert probes were regularly observed (Figs. 7G, 8E). Four of these appeared to represent complete number 7 chromosomes, whereas one was smaller and metacentric. DAPI banding (Fig. 8E, insert) and size measurement (cf. Fig. 13) were consistent with an iso(7p). This conclusion was further supported by double *in situ* hybridization experiments with biotinylated chromosome 7 inserts (detected with avidin FITC) and chromosome 7-specific alphoid AAF labeled sequences (detected with tetramethylrhodamine isothiocyanate (TRITC) conjugated second antibodies). They showed that only the four complete 7 chromosomes, contained a detectable 7 centromeric signal (metaphase, Fig. 7G,H; interphase, Fig. 7I, J). Thus, the iso(7p) marker chromosome did not have a characteristic centromeric region as it lacked both the 7 alphoid sequences and a small block of heterochromatin at 7q11 (see Fig. 8E, insert). In contrast, all four 7 chromosomes of TC 620 were labeled with the 7 alphoid probe (data not shown).

Chromosome 18

- 40 [0076] In TC 620, two apparently complete 18 chromosomes and a truncated minute chromosome were entirely decorated (Figs. 8C, D, 9). This truncated chromosome is 18q- (and possibly also 18p-). The rest of the chromosome 18 region(s) was never detected.
- 45 [0077] Three translocation chromosomes involving chromosome 18 material were typically detected, in addition to an apparently normal chromosome 18 in TC 593 metaphase spreads (Figs. 8F, G, 9). In a minor proportion of metaphases there was a small additional translocation observed. The exact chromosomal region from which this translocated 18 material derived could not be resolved by DAPI staining. The predominant karyotype for 18 is therefore close to tetrasomic in this cell line, but is under-represented in the pseudotriploid TC 620.
- 50 [0078] Both the 18q-marker chromosome in TC 620 and the three translocated 18 chromosomes in TC 593 hybridized strongly to a chromosome 18-specific alphoid repeat. Accordingly, both intact and aberrant 18 chromosomes could also be counted after *in situ* hybridization with this centromeric probe. Cremer, T. et al., *Exp. Cell Res.*, 176:199-200 (1988) (see also below). DAPI banding and hybridization to 18-specific alphoid repeats indicated that these translocation chromosomes include the entire 18q region and the centromere, with breakpoints in 18p.

55 Chromosome 22

- [0079] Two apparently normal 22 chromosomes were visualized in most TC 620 and TC 593 metaphase spreads (Fig. 10E). It was difficult to ascertain small translocations of this chromosome since hybridization with chromosome 22

inserts resulted in some cross-hybridization to other chromosomes. Some of this cross-hybridization is probably due to shared sequences from the nucleolus organizer regions (on five normal acrocentric human chromosomes) and to shared sequence motifs at the centromeres. McDermid, H.E. et al., *Chromosoma*, 94:228-234 (1986) and see Example 1. Finally, it should be noted that in contrast to conventional banding analysis, the current experimental approach clearly delineates numerical and structural chromosome aberrations in metaphase spreads of very poor quality (Fig. 10G, H) or in early prophase nuclei (Fig. 10A). These preparations are not accessible to banding analysis, as the chromosomes extensively overlie each other.

Evaluation of Chromosome Domains in Interphase Nuclei

[0080] One potential advantage of *in situ* methods is that individual human chromosomes may be directly visualized as discrete territories in interphase nuclei and thus can be of value in the analysis of solid tumor specimen. Manuelidis, L., *Hum. Genet.*, 71:288-293 (1985); Schardin, M. et al., *Hum. Genet.*, 71:281-287 (1985); Pinkel, D. et al., *Proc. Natl. Acad. Sci. USA*, 83:2934-2938 (1986). This feature of nuclear topography, also apparent in the malignant cells examined here (Figs. 7A-D, I, 10B-F), was evaluated for its accuracy and diagnostic usefulness. Figure 10A shows three apparently complete 7 chromosomes and one translocated 7p arm in a prophase TC 620 nucleus. Figures 3, and 10B show five chromosome 7 domains in interphase nuclei of TC 593, as previously depicted in metaphase spreads. Figure 10C shows a TC 620 interphase nucleus with two 18 domains of comparable sizes to those seen in normal diploid nuclei (see Example 1). A third, appreciably smaller, decorated 18 domain was also detected and represents the truncated 18 chromosomes seen in metaphase spreads described above. Figure 10D shows four chromosome 18 domains in an interphase nucleus of TC 593, which again is comparable to the numbers in metaphase nuclei. Figure 10E shows a TC 620 interphase nucleus with four chromosomes 1 domains, while Figure 10F shows a TC 593 nucleus with at least five separate chromosome 1 domains (compare Fig. 6A, B and C,D, respectively).

[0081] While the hybridization patterns of nuclei shown in Figure 10 were highly characteristic for each cell line, counts of interphase chromosome domains have some inherent difficulties. As an example, Fig. 11A (dark columns) presents an analysis of the counts of labeled interphase domains in randomly selected nuclei of diploid human lymphocytes hybridized with 7 library inserts. Although the number and relative size of chromosome specific domains can be accurately assessed in the majority of nuclei, not all nuclei present a reliable index of the chromosomal constitution, since a considerable fraction of nuclei reveal only one decorated domain and occasional nuclei show no signals. Furthermore all domains are not always cleaved separable in these 2-D preparations.

[0082] Figure 11 shows representative counts of these preparations. In agreement with TC 593 metaphase counts of chromosome 4, nuclear counts generally showed two clearly separated domains (Fig. 11E). However, the percentage of two-signal preparations was smaller in interphase than in metaphase (45.3% vs. 64%). This artificial decrease was largely due to a corresponding increased percentage of nuclei showing only one decorated domain or no signal at all. Counts of zero or one domain were not present in metaphase spreads. Significantly, 19.3% of the interphase TC 593 nuclei displayed three clearly separated chromosome 4 domains, and these extra domains were not present in interphase nuclei of diploid human lymphocytes hybridized to this or other libraries under the same conditions (Fig. 10A; Example 1). Finally, the ratio of two versus three domains was identical for both metaphase and interphase cells. Thus interphase nuclei can be reliably used for the detection of extra copies of a single chromosome or chromosomal segment but have limited reliability for detecting the loss of chromosome copies.

[0083] *In situ* hybridization of probes from subregions of interphase chromosomes may more accurately reflect general counts of chromosomal constitution than library probes (Fig. 11A), provided they are done under appropriately high stringency conditions Rappold, G. et al., *Hum. Genet.*, 67:317-325 (1984); Cremer, T. et al., *Hum. Genet.*, 74:346-352 (1986); Cremer, T. et al., *Exp. Cell Res.*, 176:199-220 (1988). However, such regional segment probes do not delineate translocated elements or aberrant chromosomes that lack this segment. Therefore such probes are also not entirely accurate. For example, counts of chromosomes 1 in TC 620 and TC 593 with a probe specific for 1q12 indicated fewer 1 chromosomes than shown here with CISS hybridization (Fig. 9). Cremer, T. et al., *Exp. Cell Res.*, 176:199-220 (1988). Counts of chromosome 7 using only a centromeric sequence further emphasize this point (see above). Double *in situ* hybridization with the AAF-modified 7 alphoid probe and biotinylated chromosome 7 library inserts typically showed interphase nuclei with five domains, of which only four were simultaneously labeled by the centromeric probe (Figs. 7I, J, 11C). In TC 620, however, both probes gave identical results (Fig. 11B).

Over Representation and Under Representation of Specific Chromosomes

[0084] The relative chromosomal dosage in these glioma lines, was also assessed with particular interest in chromosome 7, which has been noted to be generally over-represented in gliomas. Bigner, S.H. et al., *Cancer Genet. Cytogenet.* 29:165-170 (1986); Shapiro, J.R., *Semin. Oncol.*, 13:4-15 (1986). For comparison, other individual chromosome probes were used as controls. Metaphase chromosomes counts have shown that TC 620 is pseudotriploid with

a modal number of 64 chromosomes, while TC 593 is pseudotetraploid with a modal number of 83. Manuelidis, L. and Manuelidis, E.E., IN: *Progress in Neuropathology*, Vol. 4, pp 235-266, Raven Press, N.Y. (1979). Accordingly, a chromosome and its segments together would be present in a balanced state if three complete copies were present in TC 620, and four in TC 593.

5 [0085] A relative over-representation is present if more than these respective copy numbers can be demonstrated. A number lower than the expected (trisomic or tetrasomic) value indicates that the chromosome is relatively under-represented in the karyotype. In cases where additional DAPI banding information was sufficient to define the selectively decorated abnormal chromosome, the chromosome pieces labeled by the chromosome-specific inserts were put together for analysis (Fig. 9). In the second approach, computer analyses were used to independently verify these

10 results (see below).

[0086] TC 620 analyzed by banding showed the equivalent of three 1 chromosome and thus indicated a balanced state for this chromosome. The same was true for the 1p arm in TC 593 which was present in four copies. However, the distal part of 1q was under-represented in TC 593 (see the detailed description given above). In both glioma lines, 7q appeared to be balanced, while 7p was over-represented once in TC 620 and twice in TC 593. Additionally, in both glioma lines chromosomes 22 was clearly under-represented. In order to confirm this finding, double *in situ* hybridization with inserts of chromosomes 7 and 22 was performed. An example of this is shown in Fig. 12 and demonstrates over-representation of 7 DNA and under-representation of 22 DNA in the same cell. Metaphase counts done in both cell lines by this method of analysis are depicted for chromosome 7 in Fig. 11B, C (dark column) and for chromosome 22 in Fig. 11E. In summary, these two gliomas both show relative under-representation of chromosome 22 and over-representation of the 7p arm. The significant under-representation of chromosome 4 in TC 593, and a portion of 4 in TC 620 is also notable.

15 [0087] Digitized images were also used to quantitatively measure decorated areas in metaphase preparations and in interphase cells where chromosomal domains were well resolved. Quantitative evaluation of chromosome equivalents (Table 3) indicated highly concordant numbers for interphase versus metaphase in 5 of 6 examples; only in TC 20 25 593 decorated with 18 inserts was there a discrepancy. This may be due to the small sample size.

Table 3.

[0088] Twenty-four metaphase spreads showing the predominant number of chromosomes decorated with DNA 30 inserts from libraries of chromosomes 4, 7 and 18 were compared to twenty-eight interphase nuclei with well-separated domains using the same probes. Images were taken under identical (linear film) conditions and digitized. In each metaphase spread, areas obtained for each normal and aberrant chromosome were divided by the mean area obtained for n apparently complete chromosomes. In interphase nuclei, domains were compared assuming that the largest n-labeled domains represented complete (normal) interphase chromosomes. Thus the sum of these normalized values 35 represents a measure of the number of specific chromosomes equivalents in a single cell. The mean values of several cells are shown for each case. The mean numbers of chromosomes equivalents obtained for interphase and metaphase cells show a strong overall correlation coefficient of $r = + 0.95$. Compared with area measurements, the mean numbers of chromosomes equivalents determined by 2-D intensity integrals (See above) showed an overall correlation coefficient of $r = + 0.99$.

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Table 3.

| Mean number of chromosome equivalents measured by digital image analysis in malignant glioma cell lines after CISS hybridization | | | | | |
|--|------------|-----------|------------------------|------------|----------|
| | Chromosome | Cell Line | Chromosome equivalents | | |
| | | | Interphase | Methaphase | Expected |
| 50 | 4 | TC593 | 2.0 | 2.0 | 4.0 |
| | | TC620 | 2.5 | 2.4 | 3.0 |
| | 7 | TC593 | 4.4 | 4.6 | 4.0 |
| | | TC620 | 3.5 | 3.3 | 3.0 |
| 55 | 18 | TC593 | 3.0 | 3.6 | 4.0 |
| | | TC620 | 2.5 | 2.3 | 3.0 |

[0089] Chromosome equivalents derived from digital image analysis independently confirm the relative representation of target chromosomes noted in both glioma lines by DAPI banding. The segments that comprise the total metaphase signal are further detailed graphically in Fig. 13. Computer analysis was especially useful in cases where the breakpoints involved in translocated segments could not be unambiguously defined. They were also of value in a quantitative assessment of interphase-metaphase correlations, and of normal and aberrant chromosomes with distinctly different sizes.

EXAMPLE 3 Rapid Detection of Human Chromosome 21 Aberrations By In Situ Hybridization

10 DNA Probes

[0090] All plasmids contain inserts of human chromosome 21 that were mapped to 21q22.3. Moisan, J.P., Mattei, M.G., Baeteman-Volkel, M.A., Mattei, J.F., Brown, A.M.C., Garnier, J.M., Jeltsch, J.M., Masiakowsky, P., Roberts, M. & Mandel, J.L. (1985) Cytogenet. Cell Genet. 40, 701-702 (abstr.). Tanzi, R., Watkins, P., Gibbons, K., Faryniarz, A., Wallace, M., Hallewell, R., Conneally, P.M. & Gusella, J. (1985) Cytogenet. Cell Genet. 40, 760 (abstr.). Van Keuren, M.L., Watkins, P.C., Drabkin, H.A., Jabs, E.W., Gusella, J.F. & Patterson, D. (1986) Am. J. Hum. Genet. 38, 793-804. Nakai, H., Byers, M.G., Watkins, P.C., Watkins, P.A. & Shows, T.B. (1987) Cytogenet. Cell Genet. 46, 667 (abstr.). Munke, M., Foellmer, B., Watkins, P.C., Cowan, J.M., Carroll, A.J., Gusella, J.F. & Fracke, U. (1988) Am. J. Hum. Genet. 42, 542-549. All inserts were either known or verified by Southern blot analysis to be single-copy DNA: the plasmids other than pS2 are subclones derived from a λ phage library or a cosmid library. Van Keuren, M.L., Watkins, P.C., Drabkin, H.A., Jabs, E.W., Gusella, J.F. & Patterson, D. (1986) Am. J. Hum. Genet. 38, 793-804. Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A. & Chambon, P. (1982) Nucleic Acids. Res. 10, 7895-7903. Watkins, P.C., Tanzi, R.E., Gibbons, K.T., Tricoli, J.V., Landes, G., Eddy, R., Shows, T.B. & Gusella, J.F. (1985) Nucleic Acids Res. 13, 6075-6088. Watkins, P.C., Watkins, P.A., Hoffman, N. & Stanislovitis, P. (1985) Cytogenet. Cell Genet. 40, 773-774 (abstr.). The plasmids are listed in Table 4 with the Human Gene Mapping Workshop symbols and the approximate insert fragment length. Kaplan, J.C. & Carrit, B. (1987) Cytogenet. Cell Genet. 46, 257-276.

Table 4.

| Plasmids with inserts from 21q22.3 | | | | | | |
|------------------------------------|--------|------------|------------------|---------|------------------|------------|
| | | Plasmid | Insert length kb | Plasmid | Insert length kb | |
| 30 | BCEI | pS2 (23) | 0.6 | D21S56 | pPW520-10R | 4.6 |
| | D21S3 | pPW231F | 0.8 | D21S57 | pPW520-11R | 1.8 |
| | | pPW231G | 0.7 | | pPW523-10B | 6.5 |
| | D21S23 | pPW244D | 1.0 | | pPW523-1H | 7.0 |
| | D21S53 | pPW512-6B | 3.0 | | pPW523-5R | 2.2 |
| | | pPW512-8B | 3.8 | | pPW523-10R | 3.8 |
| | 40 | pPW512-1H | 2.9 | | pPW523-19R | 2.5 |
| | | pPW512-16P | 2.7 | D21S64 | pPW551-8P | 1.9 |
| | | pPW512-18P | 1.6 | | pPW551-12P | 4.2 |
| | | pPW512-4R | 4.7 | | D21S71 | pPW519-10P |
| 45 | D21S55 | pPW512-12R | 2.0 | | pPW519-11P | 3.0 |
| | | pPW518-4H | 1.6 | | pPW519-1R | 6.0 |
| | | pPW518-10P | 2.9 | | pPW519-8R | 2.9 |
| | 50 | pPW518-5R | 5.2 | | pPW519-9R | 1.7 |
| | | pPW520-5B | 5.0 | | pPW519-14R | 4.0 |
| | | pPW520-6B | 1.0 | | pPW519-22R | 1.8 |

[0091] Preparation of plasmid DNA was according to standard protocols. Maniatis, T., Fritsch, E.F. & Sambrook, J.

(1982) Molecular Cloning: A Laboratory manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY). Various probe sets were obtained by pooling plasmids (equal molar amounts), resulting in DNA probe complexities of 95 kb (all plasmids listed), 75 kb (plasmids labeled with an asterisk), or 29 kb (plasmids labeled with a dagger).

[0092] The human chromosome 21 genomic library LL21NSO2 was obtained from the American Type Culture Collection and amplified on agar plates as recommended. Phage DNA was prepared and digested with HindIII, and the DNA inserts were separated from the vector arms by preparative gel electrophoresis in 0.6% agarose. DNA was isolated from gel slices by electroelution; purified by Elutip-d chromatography. (Schleicher & Schuell); extracted with phenol/chloroform, 1:1 (vol/vol); and precipitated with ethanol.

10 Human Cells

[0093] Metaphase spreads and interphase nuclei were prepared from (i) lymphocyte cultures of normal (46, XY) individuals, (ii) lymphocytes of Down syndrome (47, +21) individuals, (iii) chorionic villi samples cultured for prenatal diagnosis (ii and iii were provided by T. Yan-Geng, Yale University Cytogenetics Laboratory), and (iv) cultures of TC620, an oligodendrogloma-derived pseudotriploid cell line. Manuelidis, L. & Manuelidis, E.E. (1979) in Progress in Neuropathology, ed. Zimmerman, H.M. (Raven, Press New York), Vol. 4, pp. 235-266. Standard techniques of colcemid treatment, hypotonic treatment, and methanol/acetic acid fixation were used. Biopsy material from the cortical region of a "normal" human brain (46, XX) was fixed, sectioned, and permeabilized as described. Manuelidis, L. & Borden, J., Chromosoma, 96:397-410(1988).

20 In situ Hybridization

[0094] Various combinations of plasmid DNA, labeled with Bioll-dUTP by nick-translation, were used for hybridization at concentrations ranging from 2 to 15 µg/ml depending on the pool size. Brigati, D.J., Myerson, D. Leary, J.J., Spalholz, B., Travis, S.Z., Fong, C.K.Y. Hsiung, G.D. & Ward, D.C. (1983) Virology 126,32-50. For example, 15 µg/ml was used when the probe mixture contained 94 kilobases (kb) of insert DNA; the probe concentration was decreased in proportion to the sequence complexity of the probe mixture. The size of the probe DNA was adjusted to a length of 150-250 nucleotides empirically by varying the DNase concentration in the nick-translation reaction. The hybridization cocktail also contained 50% formamide, 0.30 M NaCl, 0.03 M sodium citrate (pH7), 10% (wt/vol) dextran sulfate, and on occasion 0.5 mg of sonicated salmon sperm DNA per ml. Simultaneous denaturation of probe and target DNA was carried out at 75°C for 6 min (metaphase spreads) or 94°C for 11 min (tissue slices). Hybridization reactions were incubated at 37°C overnight.

[0095] Delineation of individual chromosomes with DNA probes derived from sorted human chromosomes was done by (CISS) hybridization as described above. Briefly, biotinylated chromosome 21 library DNA inserts (5µg/ml), DNase-digested human genomic DNA (200µg/ml), and salmon sperm DNA (800µg/ml) were combined in the hybridization solution, heat-denatured, and partially prehybridized for 10-30 min at 37°C before application to a separately denatured specimen.

[0096] Posthybridization washes, detection of hybridized probe by using either alkaline phosphate-conjugated avidin or fluorescein isothiocyanate-conjugated avidin, and photographic conditions were as described in Example 1. When probe sets containing 29 kb or less of target sequence were used, the fluorescein isothiocyanate detection was generally enhanced by one cycle of signal amplification as described in Example 1.

[0097] All quantitative analyses of interphase signals were carried out by using slides from several independent experiments, with more than 100 nuclei being analyzed per slide. Comparison of signals in normal and trisomic samples was done in a blind-study fashion.

[0098] This work demonstrated that CISS hybridization, under the conditions described, resulted in rapid detection of numerical and structural aberrations of chromosome 21 in both metaphase and interphase cells.

50 Use of Cloned DNA Fragments From Human Chromosome 21 to Specifically Label Chromosomes in Lymphocyte Metaphase Spreads and Interphase Nuclei

[0099] The maximal amount of unique sequence DNA in the probe set was ca94 kb; this probe set resulted in a clearly visible labeling of the terminal region of both chromatids of the chromosome 21 homologs (see Fig. 14B). These signals were seen unambiguously and without exception in all metaphase spreads, even in spreads of poor quality or from prophase cells (not shown). In normal interphase cells, the majority (65-75%) of nuclei exhibited two signals (see Fig. 14C), 25-30% showed one signal, and less than 5% showed no signal. Nuclei with three signals were found only rarely (<0.2%) and may reflect incomplete hybridization to a few tetraploid cells in the sample. Similar results were obtained with probe sets containing 29 or 75 kb of DNA. With probe sets containing fewer than 20 kb of insert DNA, there were increased numbers of cells with less than two signals. Thus, these probe sets were deemed unsuitable for

diagnostic purposes. However, such probes still yielded specific signals on the majority of chromosomes 21, even with a 6-kb single-copy DNA (see Fig. 14A), especially when signal amplification was used.

Use of chromosome library DNA CISS hybridization for detecting chromosome 21

[0100] Chromosome 21 was specifically and entirely decorated in normal lymphocyte metaphase spreads, although some additional minor binding sites were seen at or near the centromeric region of other acrocentric chromosomes, especially chromosome 13 (normal karyotype not shown; Fig. 14F). Suppression with additional DNA including a plasmid L1.26, which detects a repetitive DNA located predominantly at the centromeric region of chromosomes 13 and 21, did not efficiently suppress the minor non-21 chromosomal signals. Devilee, P., Cremer, T., Slagboom, P., Bakker, E., Schoil, H.P., Hager, H.D. Stevenson, A.F.G., Cornelisse, C.J. & Pearson, P.L. (1986) *Cytogenet. Cell Genet.* 41, 193-201. Quantitative evaluation of interphase nuclei signals again showed a negligible portion of nuclei with three signals; however, a significant increase in nuclei with less than two signals was observed (50-60% with two signals, 35-45% with one signal, and 5-10% without a signal). The numerical differences observed with the two different probes can be explained in part by the number of nuclei (up to one of three) that were excluded from the latter analysis because they exhibited larger and more diffuse signals, most likely from more than one chromosome that could not be resolved unambiguously as two separate chromosome domains in a two-dimensional representation. The minor cross-hybridizing sites noted above presented a second experimental complication but did not adversely influence data interpretation.

Testing of Cells Containing Chromosome 21 Aberrations

[0101] The optimal (94 kb) plasmid pool as well as CISS hybridization with chromosome 21 library inserts were tested further by using cells containing chromosome 21 aberrations. Both probe sets permitted a fast and unambiguous diagnosis of trisomy 21 in all metaphase spreads from Down syndrome lymphocyte cultures (see examples in Fig. 14 D and E). Furthermore, the quantitative distribution of hybridization signals in interphase nuclei of the same preparation, analyzed as described above, was similar with either type of probe [$<5\%$ of cells with no signal, 5-15% with one signal, 25-35% with two signals, and 55-65% with three signals (Fig. 14 F-J)]. Although the library DNA inserts gave up to 15% of four-signal nuclei (compare Fig. 14 F and G), most likely due to the minor binding sites on other chromosomes, the plasmid pool revealed only a negligible percentage of nuclei ($<0.2\%$) with four signals. These results indicate that trisomy 21 can be detected in a diagnostically meaningful way with small populations of nonmitotic cells.

Localization of Chromosome 21 DNA in Embryonic Chorionic Villi Cells

[0102] Embryonic chorionic villi (CV) cells were also investigated with the 94 kb plasmid probe sets in a case where the father had a reciprocal t(4;21) translocation. Hybridization to metaphase spreads of the CV cells showed that the translocated chromosome (4pter \rightarrow 4q33::21q11.2 \rightarrow 21qter) was indeed inherited by the fetus (see Fig. 13 L and M). The signals in the interphase cell nuclei (see Fig. 14K) of the CV cells had a distribution that paralleled that of cells with a normal karyotype (see above), indicating a balanced representation of 21q22.3 and excluding Down syndrome as a possible diagnosis. A small increase of nuclei with three and four signals (both $<5\%$) over that of normal lymphocytes was also observed, probably reflecting a higher portion of tetraploid cells in such CV samples.

Localization of Chromosome 21 DNA in of Glioma Tumor Cells

[0103] The diagnostic potential of the chromosome 21 probes was further tested by using a glioma tumor cell line, TC620, known to be pseudotriploid with a highly rearranged genome. Cremer, T. et al., *Exp. Cell Res.*, 176:199-220 (1988); Cremer, T. et al., *Hum. Genet.*, In Press, (1988); Manouelidis, L. and E.E. Manouelidis, In: *Progress in Neuropathology*, 4:235-266 (ed. Zimmerman, H.M.) (1979). The metaphase spreads revealed two apparently normal chromosomes 21 and one translocation chromosome (see Fig. 14 N and O). Interestingly, the chromosome 21 DNA on the translocation chromosome labeled by the library probe has a size equivalent to a normal 21q region, thus suggesting a Robertsonian translocation event. However, fine structural aberrations of 21q (i.e., small deletions, etc.) cannot be excluded by this analysis. The interphase signals seen with both the plasmid probe set and the library inserts were consistent with trisomy 21q22.3 and trisomy 21, respectively.

Localization of Chromosome 21 in DNA Sequences in Solid Tissues

[0104] The ability of the 94 kb plasmid probe set to localize chromosome 21 DNA sequences in solid tissues was also assessed. Both chromosomes 21 were clearly labeled by the probe, and located near the nucleolus; this nuclear location is consistent with the fact that chromosome 21 contains a ribosomal gene cluster that is usually localized in the

nucleolus. This observation suggests that these probes may also prove useful for evaluating the frequency of chromosome 21 mosaicism in specific cell or tissue types. In addition, it should be of interest to see if the various karyotypic changes associated with the Down syndrome phenotype alter the normal nuclear topography of chromosome 21 in neuronal tissue.

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Claims

1. A method for specifically staining or labelling any selected individual target chromosome(s) (or subregion(s) thereof) *in situ* in a sample containing chromosomal DNA from interphase cells by *in situ* hybridization in interphase cells or nuclei, comprising the steps of
 - (i) treating the sample to render the chromosomal DNA available for hybridization with complementary nucleic acid sequences,
 - (ii) hybridizing labelled probe DNA with the DNA of step (i) whereby hybridization of the probe DNA with ubiquitous repeated DNA sequences present in the DNA of step (i) (e.g. Alu and KpnI elements) is suppressed, suppression being achieved by hybridizing (e.g. by preannealing) competitor DNA which contains sequences that hybridize to the ubiquitous repeated DNA sequences with the probe DNA; wherein the probe DNA comprises DNA fragments smaller than 500 nucleotides in length.
2. The method of claim 1 for specifically staining or labelling a plurality of selected chromosomes (or subregions thereof), wherein more than one probe, each specific for a chromosome or subregion thereof and each labelled with a distinct reporter molecule, is provided.
3. The method of claim 1 or claim 2 wherein the interphase cells are tumour cells or uncultured cells from amniotic fluid.
4. The method of any one of the preceding claims, wherein the probe DNA is obtained from a chromosome-derived library.
5. The method of any one of the preceding claims, wherein the probe DNA and the competitor DNA comprise DNA fragments smaller than 500 nucleotides, for example 150-250 nucleotides in length.
6. The method of any one of the preceding claims, wherein the competitor DNA for suppression of repetitive sequences is total DNA, for example total human genomic DNA.
7. The method of any one of the preceding claims, wherein step (ii) is conducted in the presence of a carrier DNA.
8. The method of any one of the preceding claims wherein the probe DNA is labelled with: (a) avidin or biotin, (b) a fluorochrome or fluorochrome combination, or (c) an enzyme.
9. The method of claim 8(b) wherein the fluorochrome(s) are selected from fluorescein, rhodamine, Texas red, Lucifer yellow, a phycobiliprotein and a cyanin dye.

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Patentansprüche

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1. Ein Verfahren zum spezifischen Anfärben oder Markieren eines beliebig ausgewählten individuellen Zielchromosoms oder individueller Zielchromosomen (oder eines Teilbereichs oder Teilbereichen davon) *in situ* in einer Probe, die chromosomale DNS aus Interphasezellen enthält, durch *in situ* Hybridisierung in Interphasezellen oder Kernen, umfassend die Schritte:
 - (i) Behandlung der Probe, um die chromosomale DNS für eine Hybridisierung mit komplementären Nucleinsäuresequenzen verfügbar zu machen,
 - (ii) Hybridisierung markierter Sonden-DNS mit der DNS aus Schritt (i), wodurch eine Hybridisierung der Sonden-DNS mit ubiquitären repetitiven DNS-Sequenzen, die in der DNS aus Schritt (i) (z. B. Alu und KpnI Elemente) vorhanden sind, unterdrückt wird, wobei die Unterdrückung durch Hybridisierung (z.B. durch Vortempern) einer Kompetitor-DNS erreicht wird, die Sequenzen enthält, die an die ubiquitären repetitiven DNS-Sequenzen mit der Sonden-DNS hybridisieren; worin die Sonden-DNS DNS-Fragmente umfaßt, die län-

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genmäßige kleiner sind als 500 Nukleotide.

2. Das Verfahren nach Anspruch 1 zum spezifischen Anfärben oder Markieren einer Vielzahl ausgewählter Chromosomen (oder Teilbereichen davon), worin mehr als eine Sonde bereitgestellt wird, wobei jede spezifisch für ein Chromosom oder Teilbereich davon ist und jede mit einem verschiedenen Reporter-Molekül markiert ist.
- 5 3. Das Verfahren nach Anspruch 1 oder Anspruch 2, worin die Interphasezellen Tumorzellen oder nichtkultivierte Zellen aus Amnionflüssigkeit sind.
- 10 4. Das Verfahren nach einem der vorhergehenden Ansprüche, worin die Sonden-DNS aus einer von Chromosomen abstammenden Bibliothek erhalten ist.
- 15 5. Das Verfahren nach einem der vorhergehenden Ansprüche, worin die Sonden-DNS und die Kompetitor-DNS DNS-Fragmente umfassen, die längenmäßig kleiner sind als 500 Nucleotide, zum Beispiel 150-250.
6. Das Verfahren nach einem der vorhergehenden Ansprüche, worin die Kompetitor-DNS zur Unterdrückung von repetitiven Sequenzen eine Gesamt-DNS, zum Beispiel menschliche genomische Gesamt-DNS, ist.
- 20 7. Das Verfahren nach einem der vorhergehenden Ansprüche, worin Schritt (ii) in Gegenwart einer Carrier-DNS durchgeführt wird.
8. Das Verfahren nach einem der vorhergehenden Ansprüche, worin die DNS-Sonde markiert ist mit: (a) Avidin oder Biotin, (b) einem Fluorochrom oder einer Fluorochrom-Kombination, oder (c) einem Enzym.
- 25 9. Das Verfahren nach Anspruch 8(b), worin das/die Fluorochrom(e) ausgewählt ist/sind aus Fluorescein, Rhodamin, Texas Rot, Lucifergrau, einem Phycobiliprotein- und einem Cyanin-Farbstoff.

Revendications

- 30 1. Méthode pour spécifiquement colorer ou marquer *in situ* tout chromosome cible individuel sélectionné (ou une (des) sous-région(s) de celui-ci) dans un échantillon contenant de l'ADN chromosomique de cellules en interphase par hybridation *in situ* des noyaux ou cellules en interphase comprenant les étapes consistant à :
 - (i) traiter l'échantillon pour rendre l'ADN chromosomique disponible pour une hybridation avec des séquences d'acides nucléiques complémentaires ;
 - (ii) réaliser l'hybridation d'une sonde marquée avec l'ADN de l'étape (i) en supprimant l'hybridation de la sonde d'ADN avec des séquences d'ADN répétées ubiquitaires présentes dans l'ADN de l'étape (i) (exemple : des éléments Alu et KpnI), la suppression étant réalisée en hybridant (exemple : par précirculation) de l'ADN compétiteur qui contient des séquences qui hybrident aux séquences d'ADN répétées ubiquitaires avec la sonde d'ADN, ladite sonde d'ADN comprenant des fragments d'ADN de taille inférieure à 500 nucléotides.
- 35 2. Méthode selon la revendication 1, pour spécifiquement colorer ou marquer une pluralité de chromosomes sélectionnés (ou des sous-régions de ceux-ci) dans laquelle on dispose de plus d'une sonde, chacune étant spécifique pour un chromosome ou une sous-région de celui-ci et chacune étant marquée avec une molécule traceur distincte.
- 40 3. Méthode selon la revendication 1 ou 2, dans laquelle les cellules en interphase sont des cellules tumorales ou des cellules non cultivées provenant de liquide amniotique.
- 45 4. Méthode selon l'une quelconque des revendications précédentes, dans laquelle la sonde d'ADN est obtenue à partir d'une banque dérivée de chromosomes.
- 50 5. Méthode selon l'une quelconque des revendications précédentes, dans laquelle la sonde d'ADN et l'ADN compétiteur comprennent des fragments d'ADN inférieurs à 500 nucléotides, par exemple, d'une longueur de 150 à 250 nucléotides.
- 55 6. Méthode selon l'une quelconque des revendications précédentes, dans laquelle l'ADN compétiteur pour la suppression des séquences répétitives est de l'ADN total, par exemple de l'ADN génomique humain total.

7. Méthode selon l'une quelconque des revendications précédentes, dans laquelle l'étape (ii) est réalisée en présence d'un ADN porteur.
8. Méthode selon l'une quelconque des revendications précédentes, où la sonde d'ADN est marquée avec : (a) l'avidine ou la biotine, (b) un fluorochrome ou une combinaison de fluorochromes, ou (c) une enzyme.
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9. Méthode selon la revendication 8(b) dans laquelle le(s) fluorochrome(s) est(sont) choisi(s) parmi la fluorescéine, la rhodamine, le rouge Texas, le jaune Lucifer, une phycobiliprotéine et un colorant à base de cyanine.

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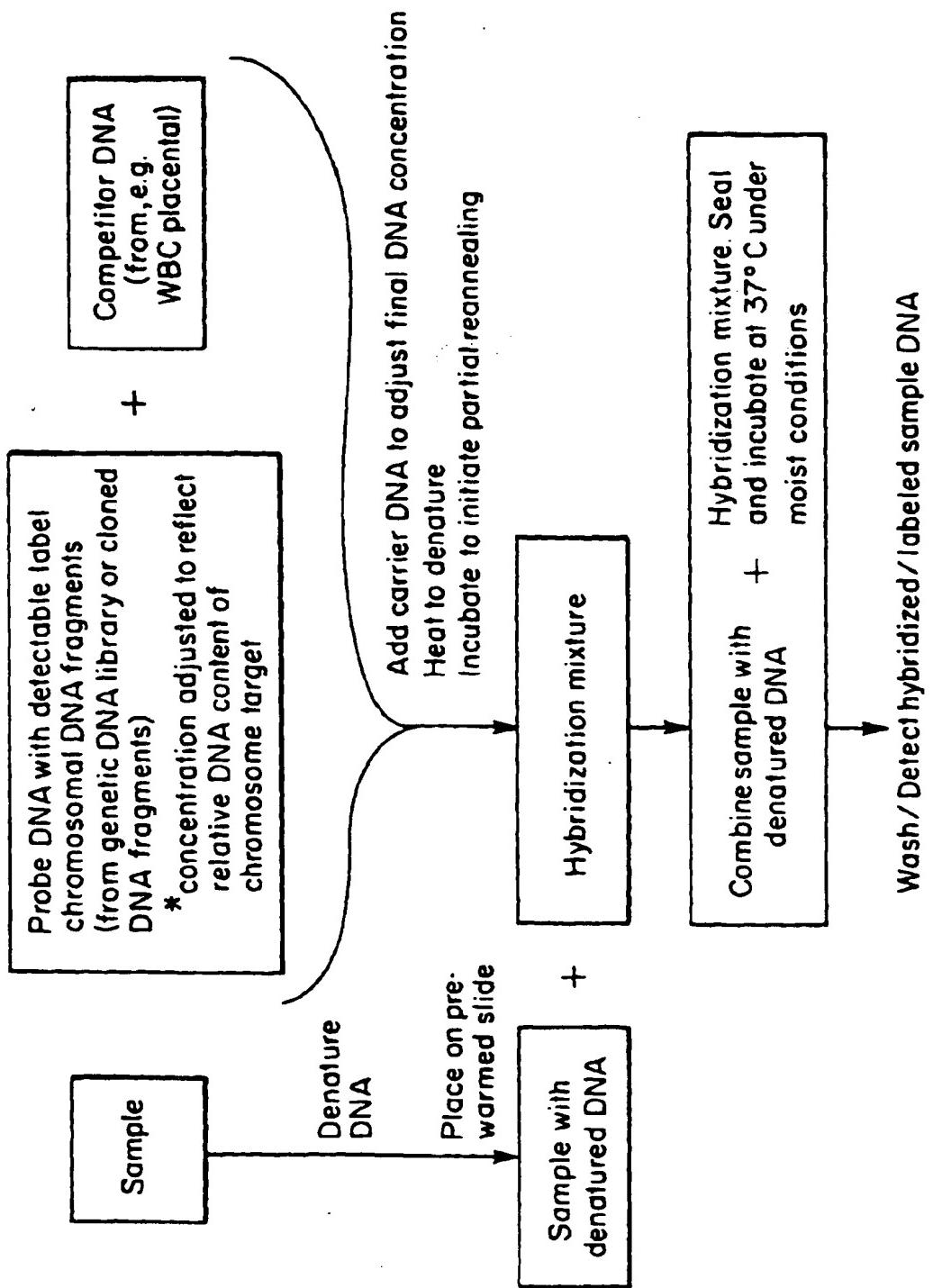


Fig. 1 OUTLINE OF CISS HYBRIDIZATION FOR SPECIFIC STAINING OF HUMAN CHROMOSOMES

FIG.2

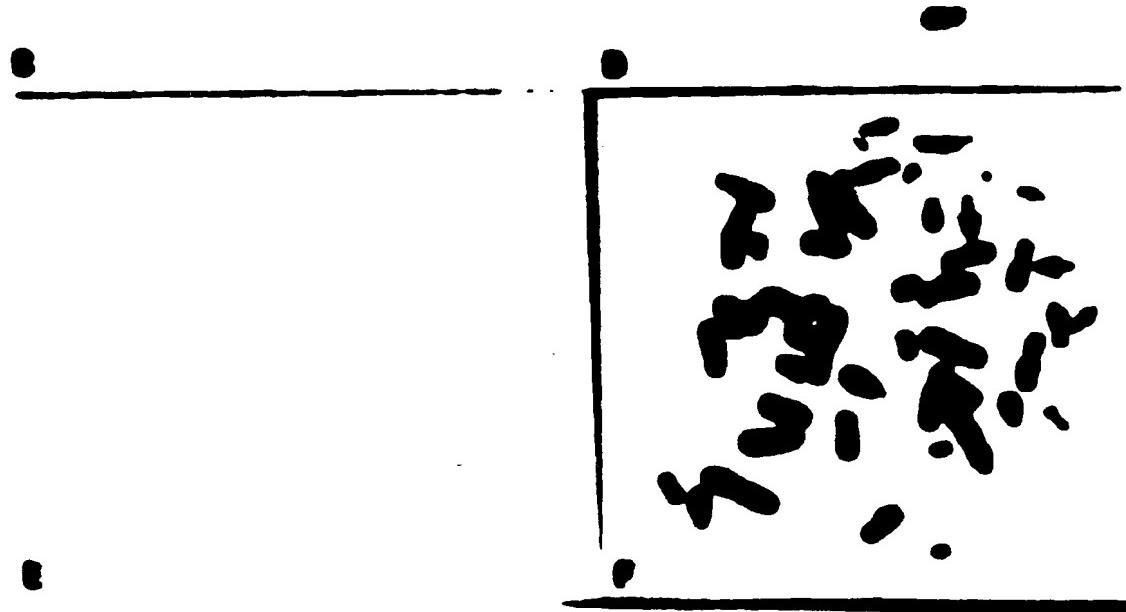
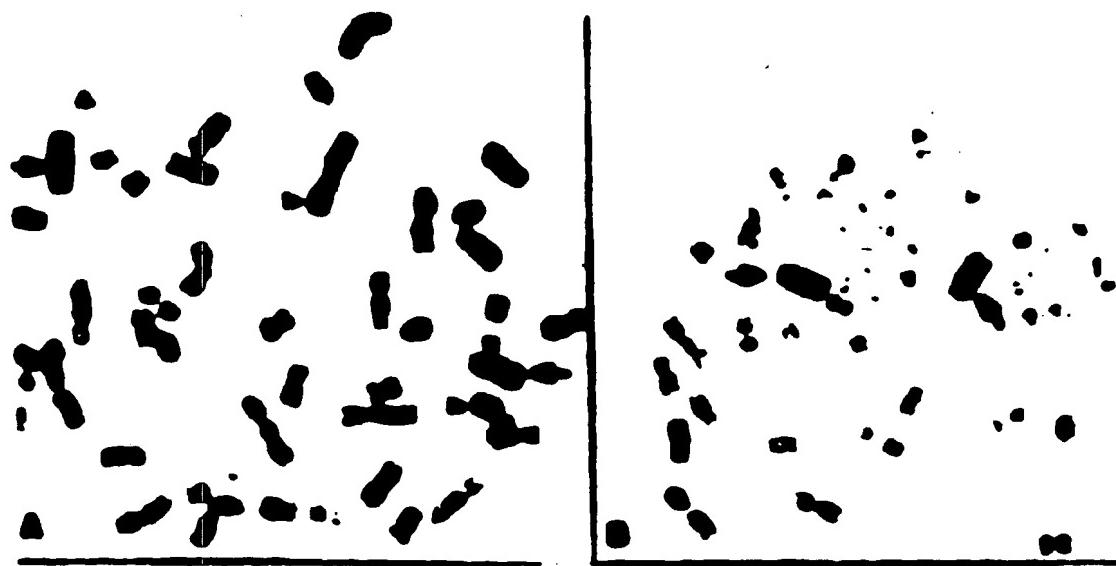


FIG.3

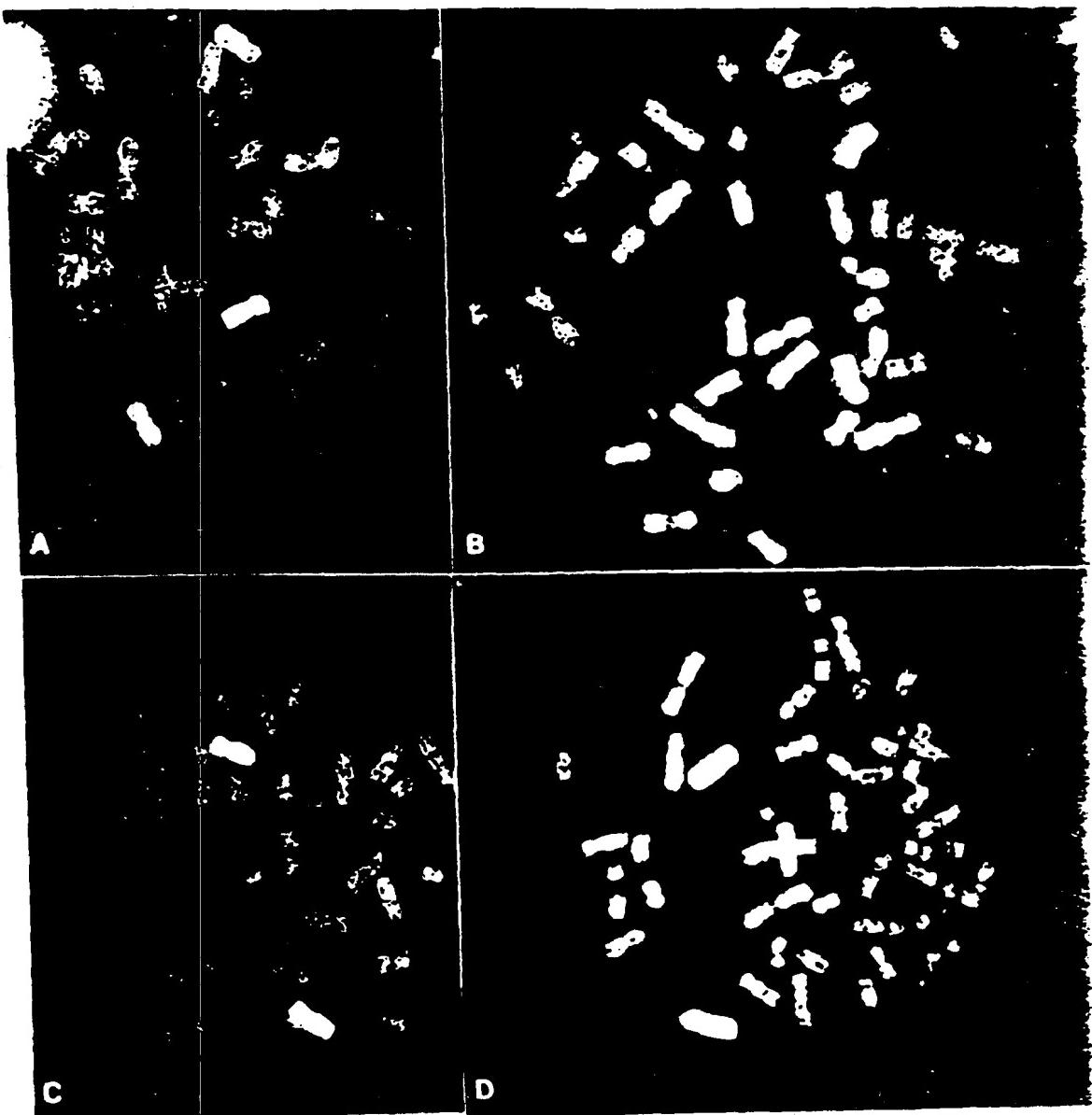


FIG.4

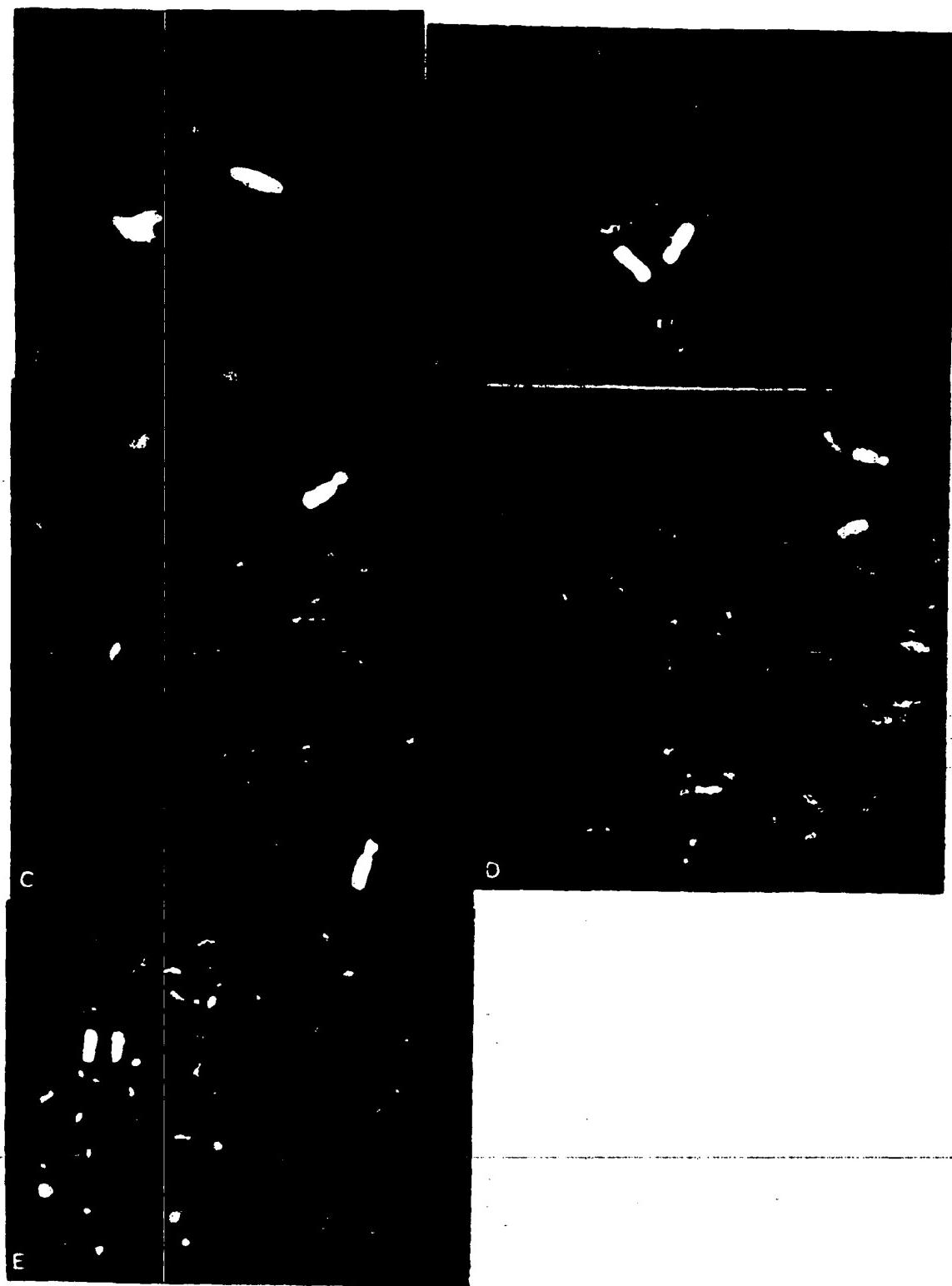


FIG.5

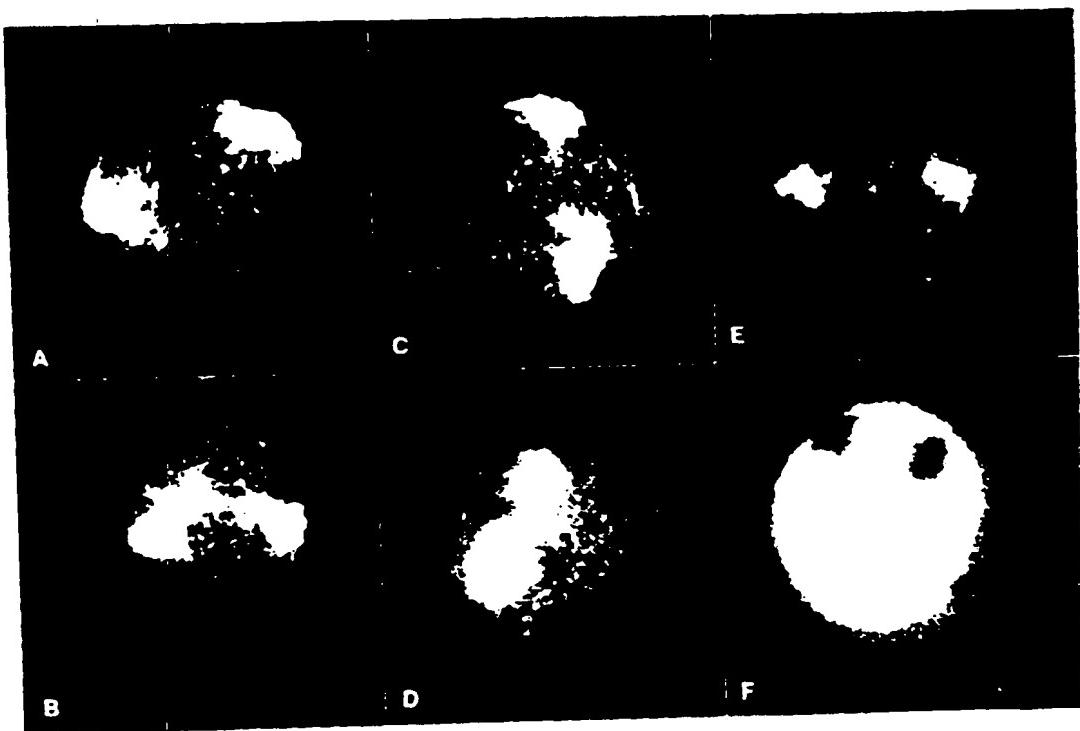


FIG.6

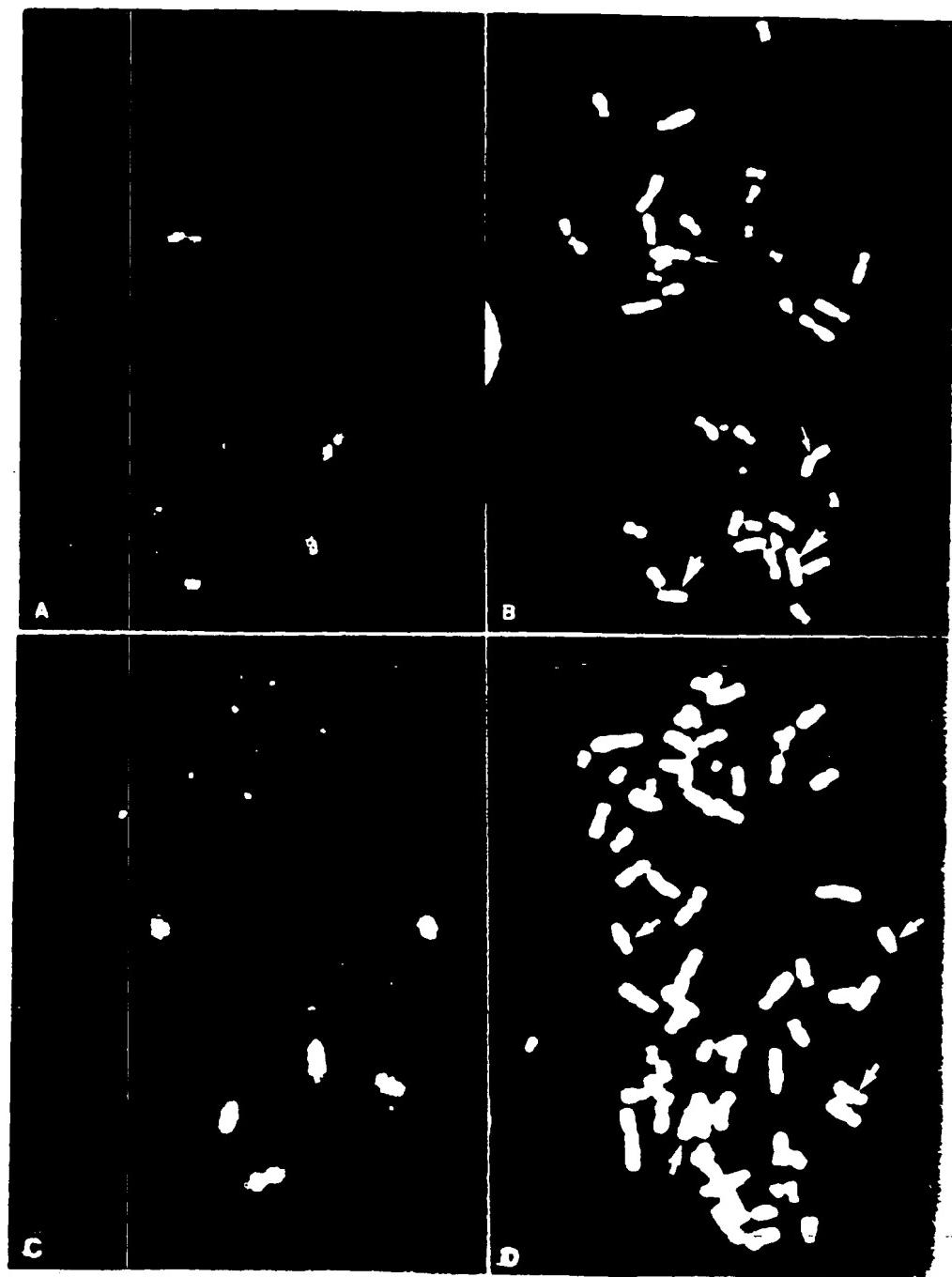


FIG.7

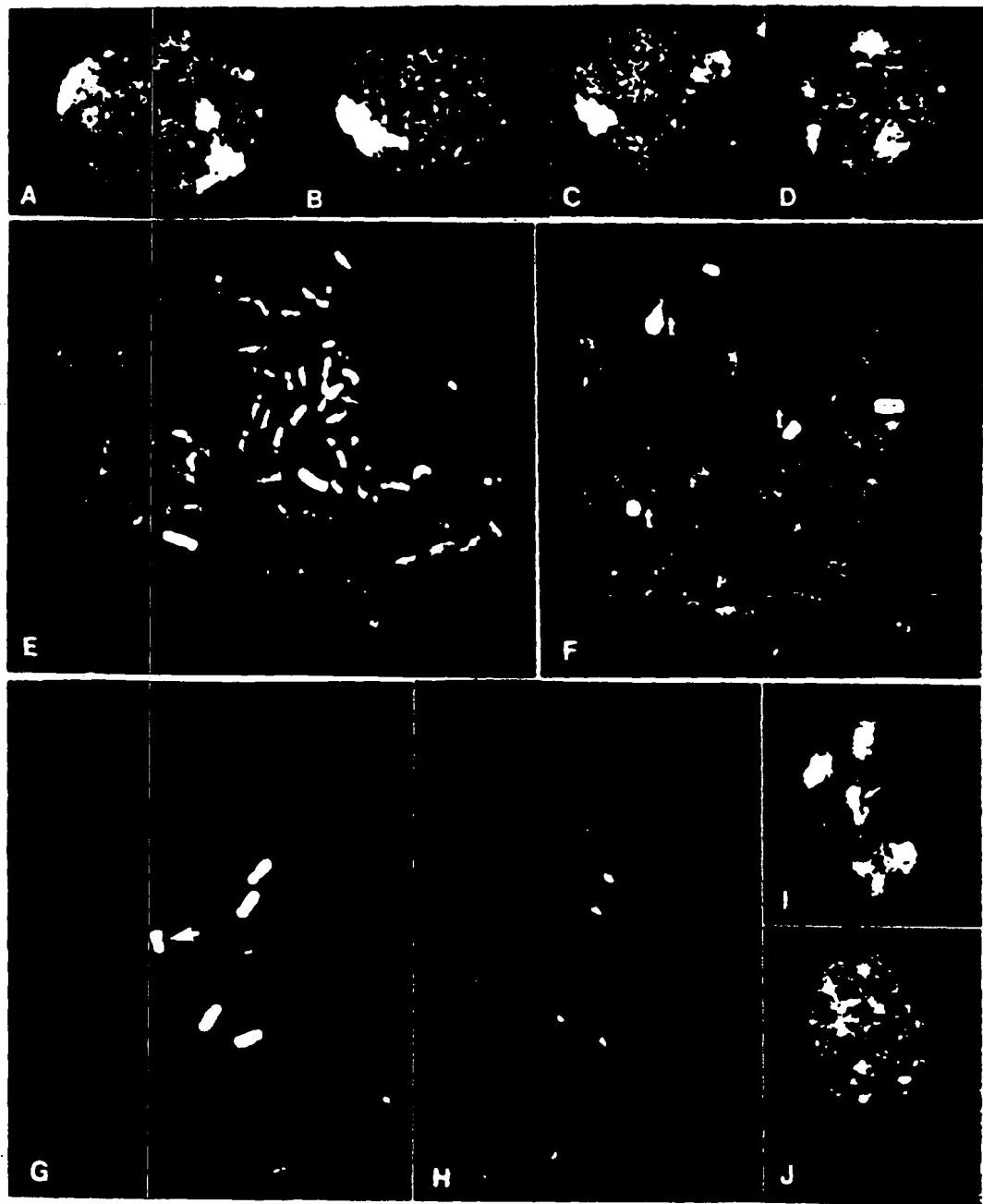


FIG. 8

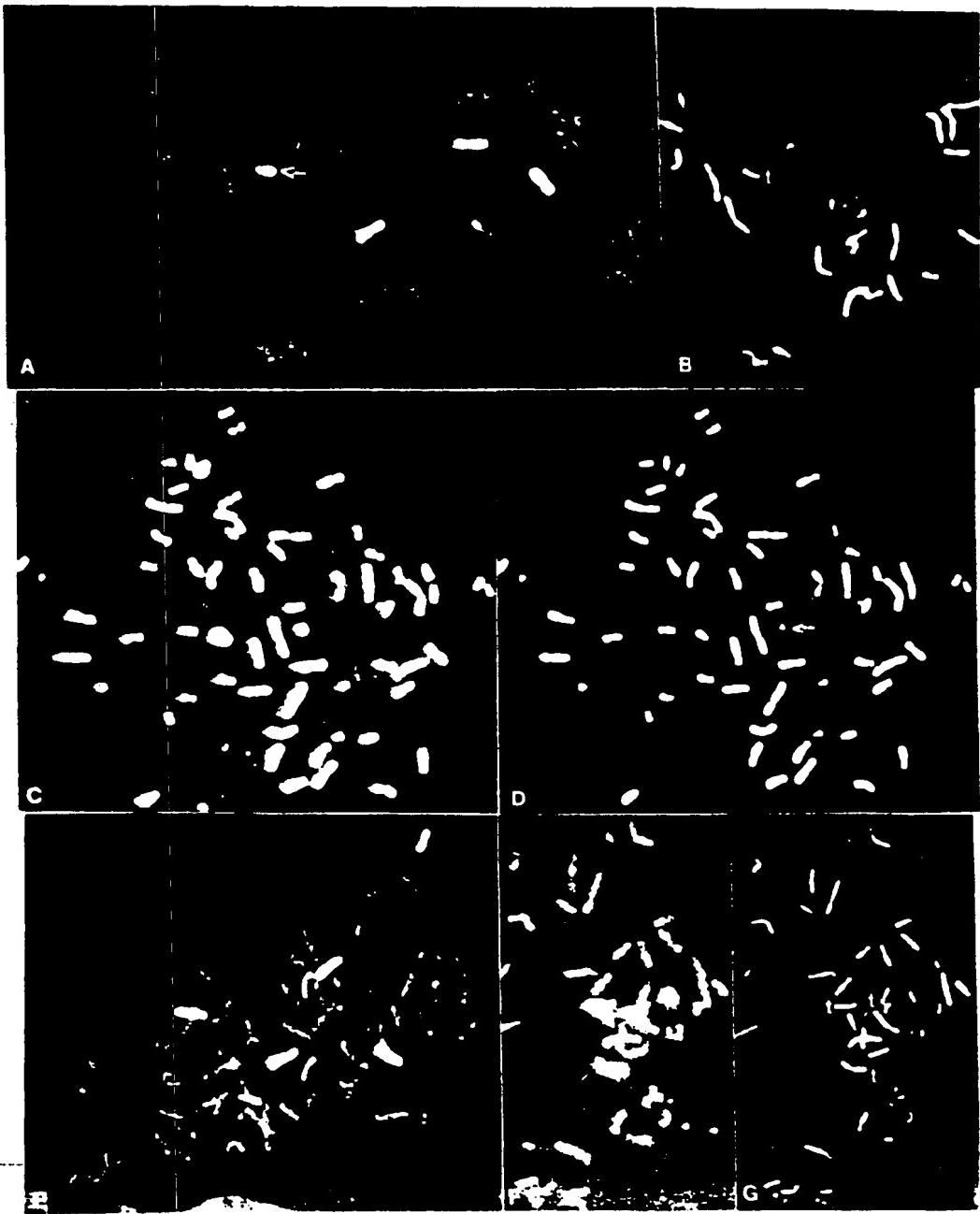


FIG. 9

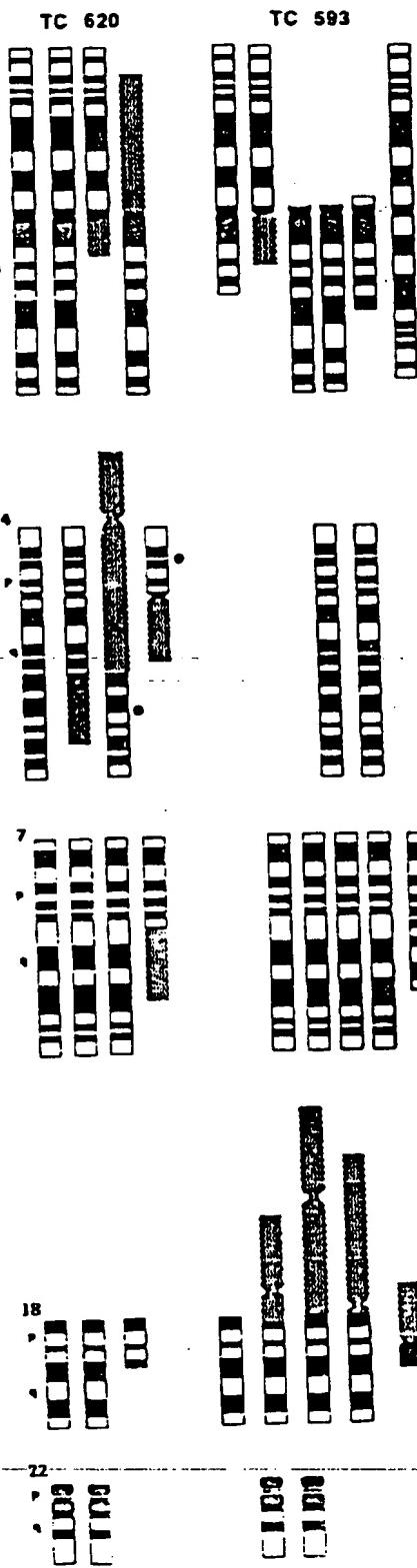
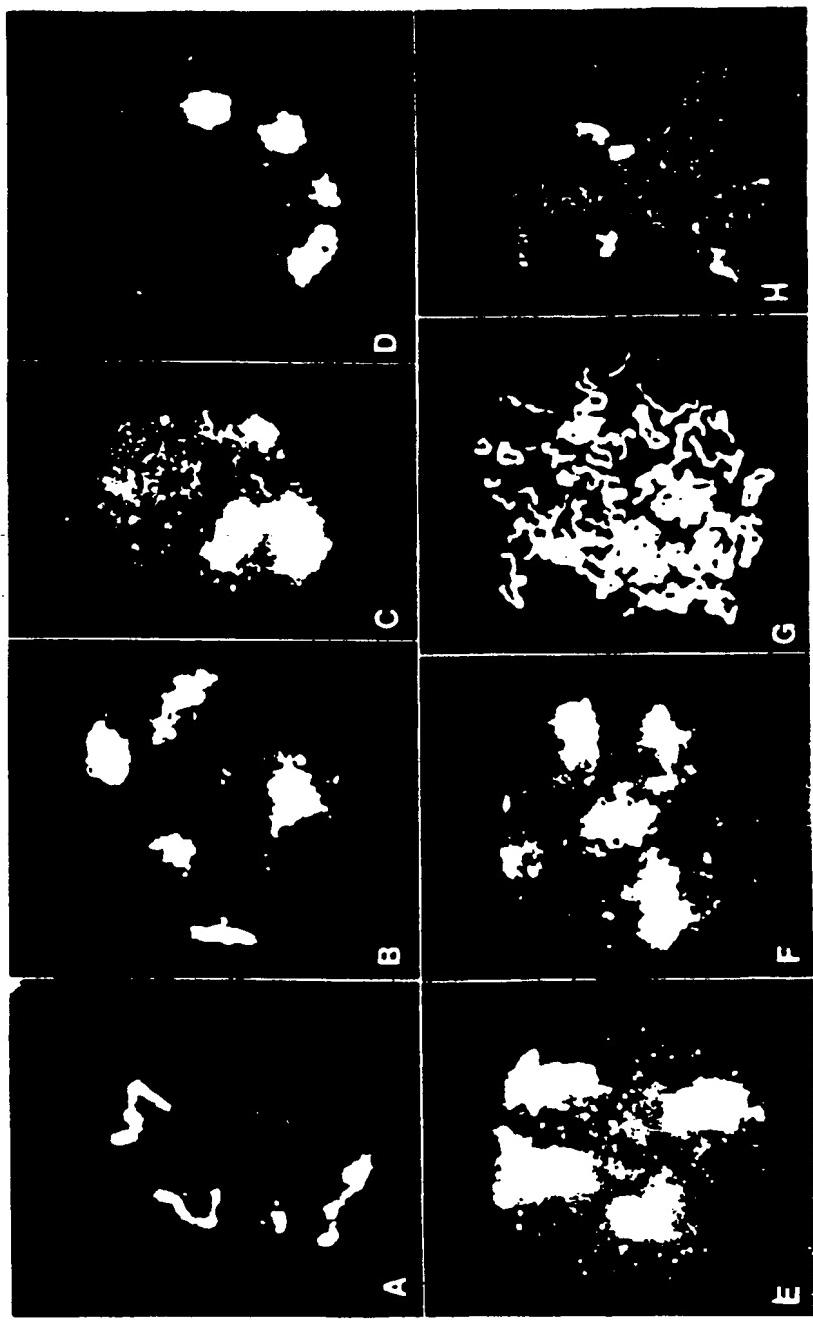


FIG.10



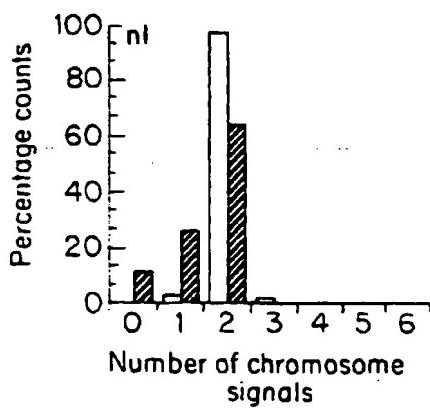


FIG. 11A

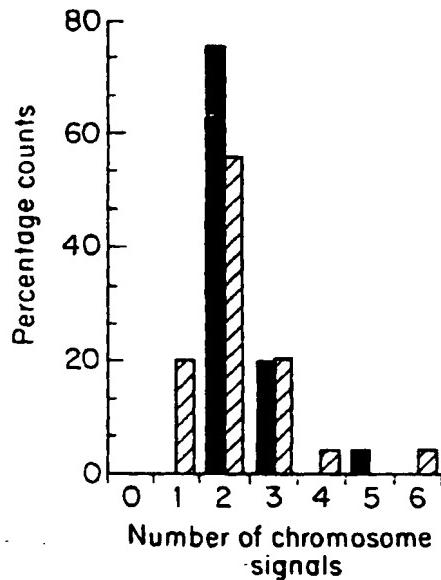


FIG. 11D

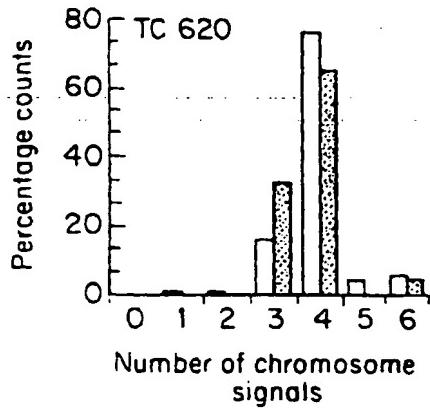


FIG. 11B

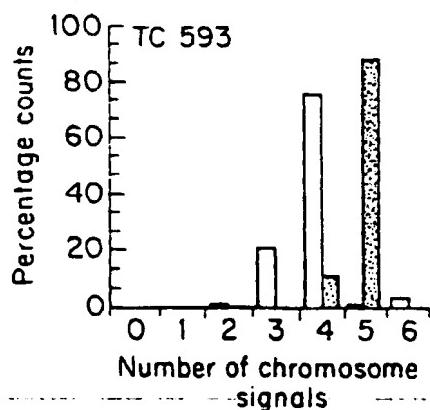


FIG. 11C

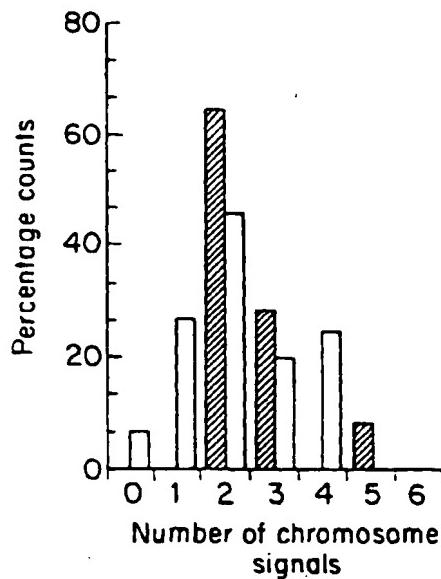


FIG. 11E

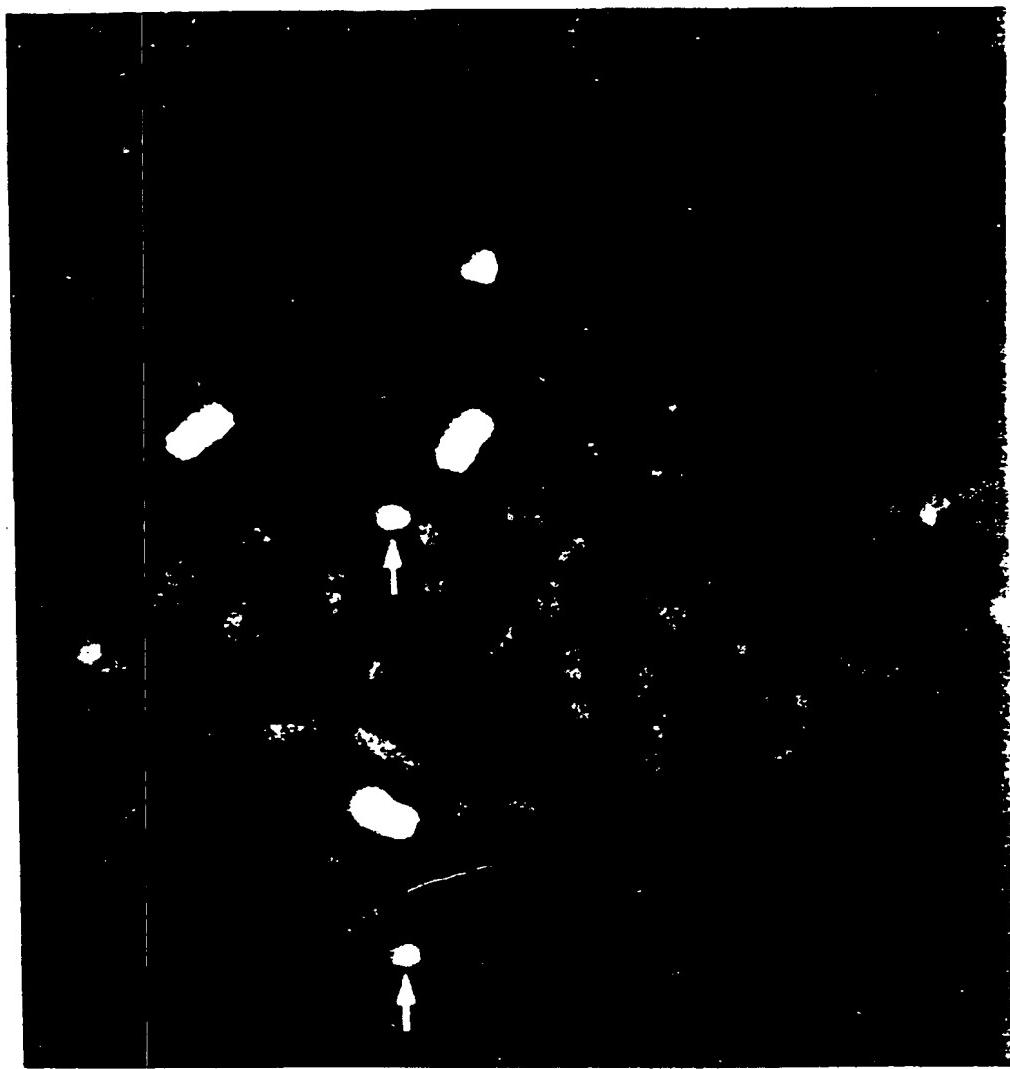


FIG.12

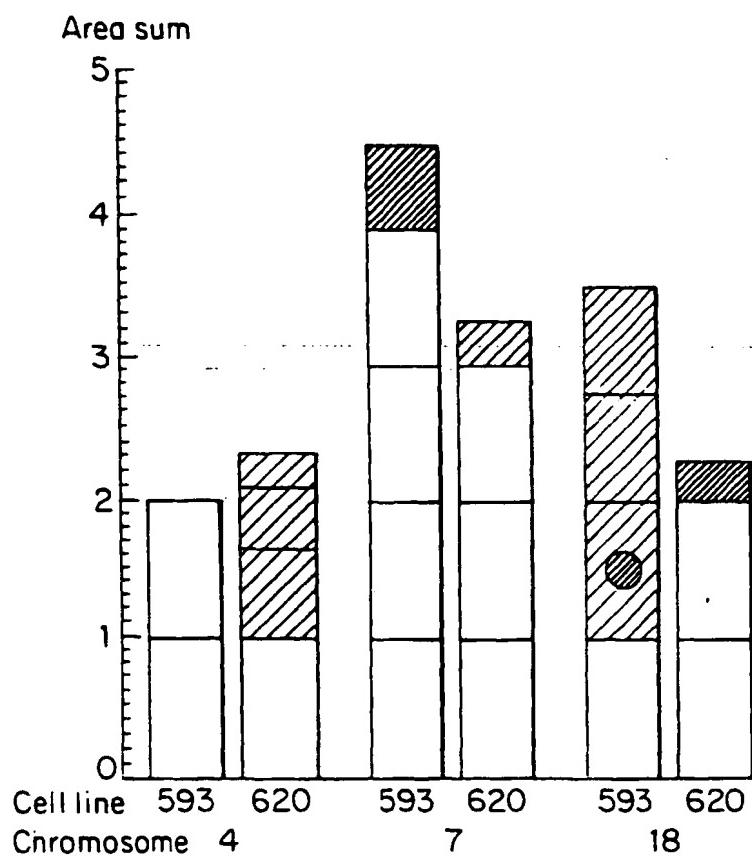
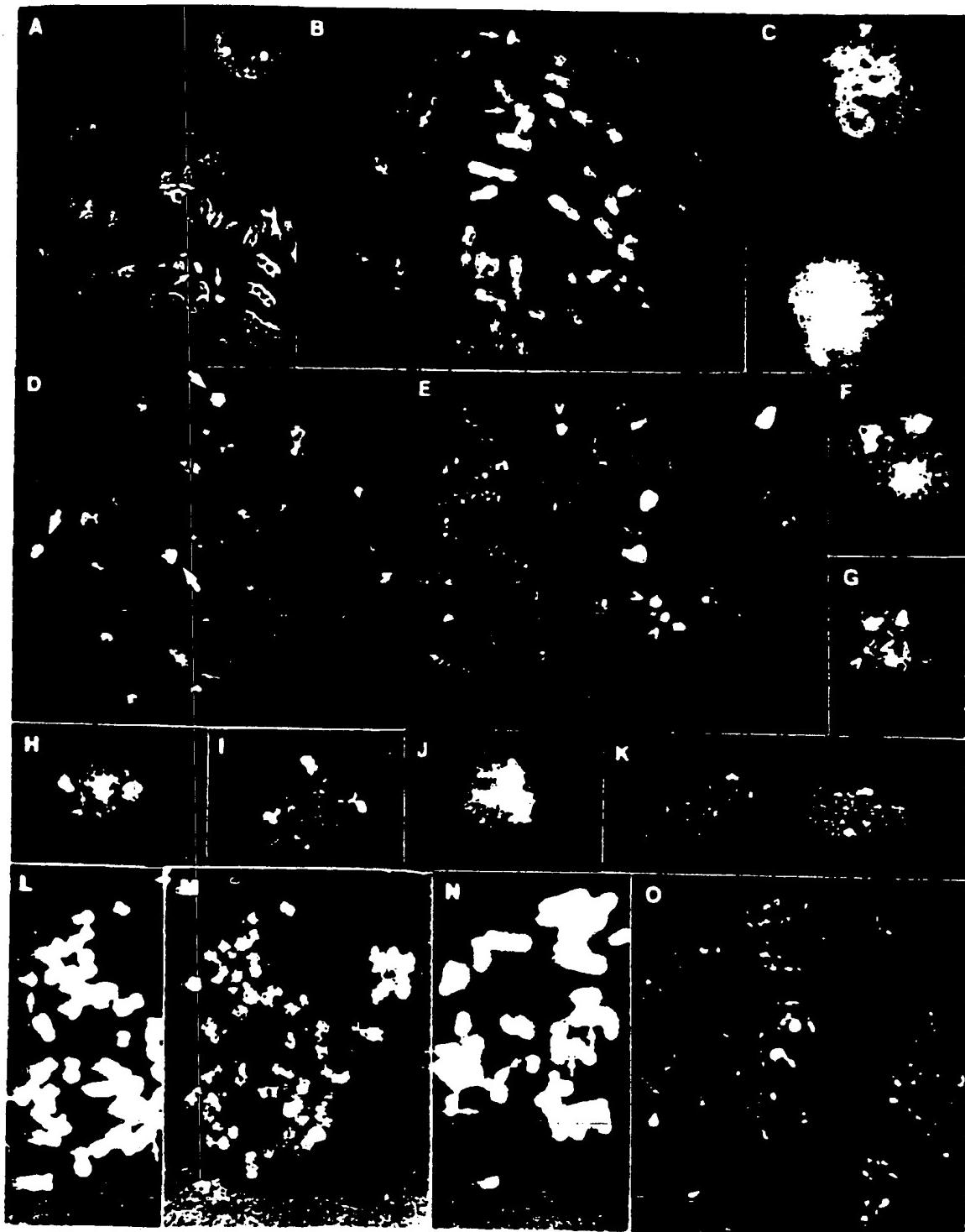


FIG. 13

FIG.14





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EP 0 637 996 B1

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Description**Background of the Invention**

5 This invention relates generally to methods and apparatus for conducting analyses. More particularly, the invention relates to the design and construction of small, typically single-use, modules capable of receiving and rapidly conducting a predetermined assay protocol on a fluid sample.

10 In recent decades the art has developed a very large number of protocols, test kits, and cartridges for conducting analyses on biological samples for various diagnostic and monitoring purposes. Immunoassays, agglutination assays, and analyses based on polymerase chain reaction, various ligand-receptor interactions, and differential migration of species in a complex sample all have been used to determine the presence or concentration of various biological compounds or contaminants, or the presence of particular cell types.

15 Recently, small, disposable devices have been developed for handling biological samples and for conducting certain clinical tests. Shoji et al. reported the use of a miniature blood gas analyzer fabricated on a silicon wafer. Shoji et al., *Sensors and Actuators*, 15:101-107 (1988). Sato et al. reported a cell fusion technique using micromechanical silicon devices. Sato et al., *Sensors and Actuators*, A21-A23:948-953 (1990). Ciba Corning Diagnostics Corp. (USA) has manufactured a microprocessor-controlled laser photometer for detecting blood clotting.

20 Micromachining technology originated in the microelectronics industry. Angell et al., *Scientific American*, 248:44-55 (1983). Micromachining technology has enabled the manufacture of microengineered devices having structural elements with minimal dimensions ranging from tens of microns (the dimensions of biological cells) to nanometers (the dimensions of some biological macromolecules). This scale is referred to herein as "mesoscale". Most experiments involving mesoscale structures have involved studies of micromechanics, i.e., mechanical motion and flow properties. The potential capability of mesoscale structures has not been exploited fully in the life sciences.

25 Brunette (*Exper. Cell Res.*, 167:203-217 (1986) and 164:11-26 (1986)) studied the behavior of fibroblasts and epithelial cells in grooves in silicon, titanium-coated polymers and the like. McCartney et al. (*Cancer Res.*, 41:3046-3051 (1981)) examined the behavior of tumor cells in grooved plastic substrates. LaCelle (*Blood Cells*, 12:179-189 (1986)) studied leukocyte and erythrocyte flow in microcapillaries to gain insight into microcirculation. Hung and Weissman reported a study of fluid dynamics in micromachined channels, but did not produce data associated with an analytic device. Hung et al., *Med. and Biol. Engineering*, 9:237-245 (1971); and Weissman et al., *Am. Inst. Chem. Eng. J.*, 17:25-30 (1971). Columbus et al. utilized a sandwich composed of two orthogonally orientated v-grooved embossed sheets in the control of capillary flow of biological fluids to discrete ion-selective electrodes in an experimental multi-channel test device. Columbus et al., *Clin. Chem.*, 33:1531-1537 (1987). Masuda et al. and Washizu et al. have reported the use of a fluid flow chamber for the manipulation of cells (e.g. cell fusion). Masuda et al., *Proceedings IEEE/IAS Meeting*, pp. 1549-1553 (1987); and Washizu et al., *Proceedings IEEE/IAS Meeting* pp. 1735-1740 (1988).

30 35 The art has not fully explored the potential of using mesoscale devices for the analyses of biological fluids and detection of microorganisms.

EP-A-483 117 discloses a capillary flow device that makes use of capillary action to draw an analyte - containing sample into a chamber for an agglutination assay.

40 The current analytical techniques utilized for the detection of microorganisms are rarely automated, usually require incubation in a suitable medium to increase the number of organisms, and invariably employ visual and/or chemical methods to identify the strain or sub-species. The inherent delay in such methods frequently necessitates medical intervention prior to definitive identification of the nature of an infection. In industrial, public health or clinical environments, such delays may have serious consequences. There is a need for convenient systems for the rapid detection of micro-organisms.

45 In some aspects, the invention provides analytical systems with optimal reaction environments that can analyze microvolumes of sample, detect substances present in very low concentrations, and produce analytical results rapidly. In other aspects, the present invention provides easily mass produced, disposable, small (e.g., less than 1 cc in volume) devices having mesoscale functional elements capable of rapid, automated analyses of preselected molecular or cellular analytes, in a range of biological and other applications. In other aspects, the present invention provides a family 50 of such devices that individually can be used to implement a range of rapid clinical tests, e.g., tests for bacterial contamination, virus infection, sperm motility, blood parameters, contaminants in food, water, or body fluids, and the like.

Summary of the Invention

55 According to aspects of the present invention, there are provided methods and devices for the detection of a pre-selected analyte in a fluid sample as set out in claims 1 and 11. The device comprises a solid substrate, typically on the order of a few millimeters thick and approximately 0.2 to 2.0 centimeters square, microfabricated to define a sample inlet port and a mesoscale flow system. The term "mesoscale" is used herein to define chambers and flow passages having cross-sectional dimensions on the order of 0.1 μm to 500 μm . The mesoscale flow channels and fluid handling

regions have a preferred depth on the order of 0.1 μm to 100 μm , typically 2 - 50 μm . The channels have preferred widths on the order of 2.0 μm to 500 μm , more preferably 3 - 100 μm . For many applications, channels of 5 - 50 μm widths will be useful. Chambers in the substrates often will have larger dimensions, e.g., a few millimeters.

The mesoscale flow system of the device includes a sample flow channel, extending from the inlet port, and an analyte detection region in fluid communication with the flow channel. The analyte detection region is provided with a binding moiety, optionally immobilized therewithin, for specifically binding the analyte. The mesoscale dimension of the detection region kinetically enhances binding of the binding moiety and the analyte. That is, in the detection region, reactants are brought close together in a confined space so that multiple molecular collisions occur. The devices may be used to implement a variety of automated, sensitive and rapid clinical tests including the analysis of cells or macromolecules, or for monitoring reactions or cell growth.

Generally, as disclosed herein, the solid substrate comprises a chip containing the mesoscale flow system. The chips are designed to exploit a combination of functional geometrical features and generally known types of clinical chemistry to implement the detection of microquantities of an analyte. The mesoscale flow system may be designed and fabricated from silicon and other solid substrates using established micromachining methods, or by molding polymeric materials. The mesoscale flow systems in the devices may be constructed by microfabricating flow channel(s) and detection region(s) into the surface of the substrate, and then adhering a cover, e.g., a transparent glass cover, over the surface. The channels and chambers in cross-section taken through the thickness of the chip may be triangular, trapezoidal, square, rectangular, circular, or any other shape. The devices typically are designated on a scale suitable to analyze microvolumes (<5 μL) of sample, introduced into the flow system through an inlet port defined, e.g., by a hole communicating with the flow system through the substrate or through a transparent coverslip. Cells or other analytes present in very low concentrations (e.g. nanogram quantities) in microvolumes of a sample fluid can be rapidly analyzed (e.g., <10 minutes).

The chips typically will be used with an appliance which contains a nesting site for holding the chip, and which mates an input port on the chip with a flow line in the appliance. After biological fluid such as blood, plasma, serum, urine, sputum, saliva, or other fluids suspected to contain a particular analyte, cellular contaminant, or toxin is applied to the inlet port of the substrate, the chip is placed in the appliance and a pump is actuated to force the sample through the flow system. Alternatively, a sample may be injected into the chip by the appliance, or the sample may enter the mesoscale flow system of the chip through the inlet port by capillary action.

In the devices, the binding of an analyte to a binding moiety serves as a positive indication of the presence of the analyte in a sample. The mesoscale detection region is provided with a binding moiety capable of specifically binding to the preselected analyte. The binding moiety may be delivered to the detection region in, e.g., a solution. Alternatively, the binding moiety may be immobilized in the detection region. The internal surfaces of the mesoscale detection region of the device may be coated with an immobilized binding moiety to enable the surface to interact with a fluid sample in order to detect or separate specific fluid sample constituents. Antibodies or polynucleotide probes may be immobilized on the surface of the flow channels, enabling the use of the mesoscale flow systems for immunoassays or polynucleotide hybridization assays. The binding moiety also may comprise a ligand or receptor. A binding moiety capable of binding cells via a cell surface molecule may be utilized, to enable the isolation or detection of a cell population in a biological microsample. The mesoscale flow system may also include protrusions or a section of reduced cross sectional area to enable the sorting or lysis of cells in the microsample upon flow through the flow system.

Analyte binding to a binding moiety in the detection region may be detected optically, e.g., through a transparent or translucent window, such as a transparent cover over the detection region or through a translucent section of the substrate itself. Changes in color, fluorescence, luminescence, etc., upon binding of the analyte and the binding moiety, indicating a positive assay can be detected either visually or by machine. The appliance may include sensing equipment, such as a spectrophotometer, capable of detecting changes in optical properties, due to the binding of an analyte to a binding moiety in the detection region, through a clear cover disposed over the detection region.

The device may further include means for delivering reagents such as a labeled substance to the detection region that binds to the analyte to provide a detectable signal indicative of the presence of the analyte. Optionally, depending on the protocol being exploited in the structure of the chip, the appliance also may be designed to inject reagents necessary to complete the assay, e.g., to inject a binding protein tagged with an optically detectable moiety, a substrate solution for reaction with an enzyme, or other reagents.

A positive assay may also be indicated by detectable agglutination or flow impedance upon analyte binding. The presence of a preselected analyte in a fluid sample may be detected by sensing analyte-induced changes in sample fluid flow properties, such as changes in the pressure or electrical conductivity, at different points in the flow system. In one embodiment, analyte induced restriction or blockage of flow in the mesoscale flow system, e.g., in the fractal region, may be detected by pressure detectors, e.g., in the appliance used in combination with the device. In another embodiment, analyte-induced changes in conductivity in a region of the flow system caused by introduction of a sample fluid may be readily detected through electrical conductivity sensors in contact with the flow system. For example, the presence of analyte may cause clogging of a restricted flow passage, and beyond the passage, the absence of liquid can be detected by measuring conductivity. The appliance also may include electrical contacts in the nesting region

which mate with contacts integrated into the structure of the chip to, e.g., provide electrical resistance heating or cooling to a portion of the flow system, or receive electrical signals indicative of a pressure reading, conductivity, or the like, sensed in some region of the flow system to indicate (flow restriction, as a) positive indication of the presence of the analyte.

5 The mesoscale devices can be adapted to perform a wide range of biological or other tests. A device may include two or more separated flow systems, e.g., fed by a common inlet port, with different binding moieties in, e.g., different detection regions to enable the detection of two or more analytes simultaneously. The device may also comprise a control flow system so that data from the sample region and the control region may be detected and compared. Essentially any test involving detection of the presence or concentration of a molecular or atomic scale analyte, or the presence of
10 a particular cell type, can be implemented to advantage in such structures. The mesoscale devices may provide a rapid chemical test for the detection of pathogenic bacteria or viruses. The devices may also provide a rapid test for the presence or concentration of blood constituents such as hormones. Other applications include but are not limited to a range of other biological assays such as blood type testing.

The devices as disclosed herein are all characterized by a mesoscale detection region containing a binding moiety
15 that reacts with the analyte component, such as a molecular analyte or a cell type, to detect the presence or concentration of the analyte. The device may be readily sterilized prior to an assay. Assays may be completed rapidly, and at the conclusion of the assay the chip can be discarded, which advantageously prevents contamination between samples, entombs potentially hazardous material, produces only microvolumes of waste fluid for disposal, and provides an inexpensive, microsample analysis. Some of the features and benefits of the devices are summarized in Table 1.
20

TABLE 1

| Feature | Benefit |
|--------------------------------|--|
| Flexibility | No limits to the number of chip designs or applications available. |
| Reproducible | Allows reliable, standardized, mass production of chips. |
| Low Cost Production | Allows competitive pricing with existing systems. Disposable nature for single-use processes. |
| Small Size | No bulky instrumentation required. Lends itself to portable units and systems designed for use in non-conventional lab environments. Minimal storage and shipping costs. |
| Microscale | Minimal sample and reagent volumes required. Reduces reagent costs, especially for more expensive, specialized test procedures. Allows simplified instrumentation schemes. |
| Sterility | Chips can be sterilized for use in microbiological assays and other procedures requiring clean environments. |
| Sealed System | Minimizes biohazards. Ensures process integrity. |
| Multiple Circuit Capabilities | Can perform multiple processes or analyses on a single chip. Allows panel assays. |
| Multiple Detector Capabilities | Expands capabilities for assay and process monitoring to virtually any system. Allows broad range of applications. |
| Reuseable Chips | Reduces per process cost to the user for certain applications. |

Brief Description of the Drawings

50 FIGURE 1 is a schematic longitudinal cross sectional view of a device according to the invention that includes a solid substrate 14, on which are machined entry ports 16 connected by mesoscale flow channel 20, with a transparent cover 12 adhered to the surface of the substrate.

FIGURE 2 is a perspective view of the device of Figure 1.

55 FIGURE 3 is a cross sectional view of a support block 30 for holding device 10 that includes ports 32 for delivery or removal of reagents or sample fluids from device 10.

FIGURE 4 is a schematic illustration of analytical device 10 nested within appliance 50, which is used to support the device 10 and to regulate and detect the pressure of sample fluids in device 10.

FIGURES 5A-D are schematic illustrations of a cross-section of a portion of a mesoscale flow channel 20 within a

substrate 14, on which antibodies 103 are immobilized, and illustrating changing states of the system during an analysis.

FIGURES 6A-D are schematic illustrations of a cross-section of a portion of a mesoscale flow channel 20 within a substrate 14, on which DNA binding probes 110 are immobilized, and illustrating changing states of the system during an analysis.

FIGURE 7 is a cross sectional perspective view of a flow channel 20 on the inert substrate 14 with cell or debris filtering protrusions 122 extending from a wall of the flow channel.

FIGURE 8 is a cross sectional view of a flow channel 20 on the inert substrate 14 with cell piercing protrusions 124 extending from a wall of the channel.

FIGURE 9 is a schematic top view of a analytical device fabricated with a series of mesoscale chambers suitable for implementing a variety of functions including cell sorting, cell lysing and PCR analysis.

FIGURE 10a is a schematic longitudinal cross sectional view of a device according to the invention which includes electrical contacts 17 and 18 for measuring conductivity of fluids in the device.

FIGURE 10b is a perspective view of the device shown in Figure 10a.

FIGURE 11 is a schematic plan view of a substrate microfabricated with a pair of fractally bifurcating flow channels 40.

FIGURE 12 is a schematic perspective view of an apparatus 60 used in combination with device 10 for viewing the contents of device 10.

FIGURE 13 is a schematic cross sectional view of the apparatus 60 of Figure 12.

FIGURE 14 is a schematic plan view of device 10 microfabricated with a mesoscale flow system that includes the tortuous channels 22A and 22B which allow the timed addition and mixing of assay components during an assay.

Like reference characters in the respective drawn figures indicate corresponding parts.

Detailed Description

The invention provides a family of small, mass produced, typically one-use devices for detecting a particular analyte in a fluid microsample. The device comprises a solid substrate, typically on the order of a few millimeters thick and approximately 0.2 to 2.0 centimeters square, that is microfabricated to define a sample inlet port and a mesoscale flow system. The mesoscale flow system includes at least one sample flow channel extending from the inlet port and at least one analyte detection region in fluid communication with the flow channel which contains a binding moiety for specifically binding the analyte. Optionally the binding moiety may be immobilized within the detection region. As disclosed herein, mesoscale detection systems may be used in a wide range of rapid tests, including the analysis of cells or macromolecules, or for monitoring reactions or cell culture growth. The devices may be fabricated with two or more mesoscale flow systems which comprise two or more different detection regions containing binding moieties for different analytes, allowing two or more assays to be conducted simultaneously. At the conclusion of the assay the devices typically are discarded.

Mesoscale devices having flow channels and chambers with at least one mesoscale dimension can be designed and fabricated in large quantities from a solid substrate material. Silicon is preferred because of the enormous body of technology permitting its precise and efficient fabrication, but other materials may be used including polymers such as polytetrafluoroethylenes. The sample inlet port, the mesoscale flow system, including the sample flow channel(s) and the analyte detection region(s), and other functional elements thus may be fabricated inexpensively in large quantities from a silicon substrate by any of a variety of micromachining methods known to those skilled in the art. The micromachining methods available include film deposition processes such as spin coating and chemical vapor deposition, laser fabrication or photolithographic techniques such as UV or X-ray processes, or etching methods including wet chemical processes or plasma processes. (See, e.g., Manz et al., *Trends in Analytical Chemistry* 10: 144-149 (1991)). Flow channels of varying widths and depths can be fabricated with mesoscale dimensions, i.e., with cross-sectional dimensions on the order of 0.1 to 500 μm .

The silicon substrate containing a fabricated mesoscale flow channel may be covered and sealed with a thin anodically bonded glass cover. Other clear or opaque cover materials may be used. Alternatively, two silicon substrates can be sandwiched, or a silicon substrate may be sandwiched between two glass covers. The use of a transparent cover results in a window which facilitates dynamic viewing of the channel contents and allows optical probing of the detection region either visually or by machine. Other fabrication approaches may be used. In one embodiment, electron micrographs of biological structures such as circulatory networks may be used as masks for fabricating mesoscale flow systems on the substrate. Mesoscale flow systems may be fabricated in a range of sizes and conformations.

In one embodiment, illustrated schematically in Figures 1 and 2, the device 10 may include a silicon substrate 14 microfabricated with a mesoscale flow channel 20, which, in this instance, also serves as a detection region, and which may be provided with binding moieties capable of binding a preselected analyte. Sample or reagent fluid may be added or recovered from flow channel 20 via ports 16 which are fabricated on either end of the flow channel 20. The substrate 14 is covered with a glass or plastic window 12. During an analysis, the device 10 may be placed in support structure

30 (Figure 3), which is provided with internal flow paths 32 for delivery and recovery of sample fluid through the inlet ports of device 10. The dimensions of the microchannels in the silicon mesoscale devices may vary in the range from approximately 2.0 μm - 500 μm wide and approximately 0.1 μm - 500 μm in depth, a range comparable to cellular or macromolecular dimensions, where fluid motion of multiphasic materials such as fluid and cell culture medium has not been systemically investigated. The inlet ports on the devices may be microfabricated with mesoscale or, alternatively, larger dimensions.

The capacity of the devices is very small and therefore reduces the amount of sample fluid required for an analysis. For example, in a 1 cm x 1 cm silicon substrate, having on its surface an array of 500 grooves which are 10 microns wide x 10 microns deep x 1 cm (10^4 microns) long, the volume of each groove is $10^{-3} \mu\text{L}$ and the total volume of the 500 grooves is 0.5 μL . The low volume of the mesoscale flow systems enhances the reaction rates of assays conducted in the devices. For example, in a mesoscale detection chamber containing a surface coating of an immobilized binding moiety, as predicted by the Law of Mass Action, as the volume of the mesoscale detection chamber decreases, the surface area to volume ratio of the binding moiety in the detection region increases, which results in an increased rate of intermolecular reaction between the analyte and the binding moiety. The entire mesoscale flow systems of devices of the invention typically have volumes on the order of less than 10 μL . Detection chambers are small enough in at least one dimension to favor fast kinetics. The mesoscale flow systems in the devices may be microfabricated with microliter volumes, or alternatively nanoliter volumes or less, which advantageously limits the amount of sample and/or reagent fluids required for an assay.

The analytical devices containing the mesoscale flow system can be used in combination with an appliance for delivering and receiving fluids to and from the devices, such as appliance 50, shown schematically in Figure 4, which incorporates a nesting site 58 for holding the device 10, and for registering ports, e.g., ports 16 on the device 10, with a flow line 56 in the appliance. The appliance may include means, such as pump 52 shown in Figure 5, for forcing the sample through the flow system. After a biological fluid sample suspected to contain a particular analyte is applied to the inlet port 51 of the appliance, pump 52 is actuated to force the sample into port 16 of device 10 and the mesoscale flow channel 20. Alternatively a sample may be injected into the chip by the appliance, or the sample may enter the mesoscale flow system of the device through the inlet port by capillary action. In another embodiment, the appliance may be disposed over the substrate, and may be provided with a flow line communicating with the inlet ports in the device, e.g., in the absence of a cover over the device, to enable a sample to be injected via the appliance into the device. Other embodiments of appliances may be fabricated in accordance with the invention for use in different assay protocols with different devices. The flow systems of the devices may be filled to a hydraulically full volume and the appliance may be utilized to direct the flow of fluid through the flow system, e.g., by means of valves located in the device or in the appliance.

The analytical devices also may be utilized in combination with an appliance for viewing the contents of the mesoscale channels in the devices. The appliance in one embodiment may comprise a microscope for viewing the contents of the mesoscale channels in the devices. In another embodiment, a camera may be included in the appliance, as illustrated in the appliance 60 shown schematically in Figures 12 and 13. The appliance 60 is provided with a housing 62, a viewing screen 64 and a slot 66 for inserting a chip into the appliance. As shown in cross section in Figure 20, the appliance 60 also includes a video camera 68, an optical system 70, and a tilt mechanism 72 for holding device 10, and allowing the placement and angle of device 10 to be adjusted manually. The optical system 70 may include a lens system for magnifying the channel contents, as well as a light source. The video camera 68 and screen 64 allow analyte induced changes in sample fluid properties, such as flow properties or color, to be monitored visually, and optionally recorded using the appliance.

Binding moieties may be introduced into the mesoscale detection region in a solution via an inlet port in fluid communication with the detection region. Alternatively, binding moieties may be immobilized in the mesoscale detection region of the analytical devices after its manufacture by, for example, physical absorption or chemical attachment to the surface of the flow system or to a solid phase reactant such as a polymeric bead disposed in the detection region.

The surfaces of the mesoscale detection channels in the silicon substrates can be chemically activated and reacted with a protein, lipid, polysaccharide or other macromolecule to form a coated surface in the mesoscale flow channels. Techniques for the chemical activation of siliceous surfaces are available in the art. (See, e.g., Haller in: Solid Phase Biochemistry, W.H. Scouten, Ed., John Wiley, New York, pp 535-597 (1983); and Mandenius et al., Anal. Biochem., 137:106-114 (1984) and 170: 68-72 (1988) and Mandenius et al., Methods in Enzymology, 137: 388-394). There are a number of techniques in the art for attaching biomolecules to silicon. For example, enzymes may be immobilized on silicon devices via entrapment in a photo-crosslinkable polyvinyl alcohol (Howe et al., IEEE Transactions Electron Devices, ED33:499-506 (1986) or attached indirectly using preformed membranes (Hanazato et al., IEEE Transactions Electron Devices, ED33:47-51 (1986). A hydrophobic bilayer glycerol monooleate coating may be fabricated on a silicon substrate. Fromherz et al., Biochim. Biophys. Acta, 1062:103-107 (1991).

Protein conjugation and immobilization techniques known in the art may be adapted for use with activated siliceous surfaces. Kennedy et al., Clin. Chem. Acta, 70:1-31 (1976); Sankolli et al., J. Imm. Methods, 104:191-194 (1987); Kricka et al., Clin. Chem., 26:741-744 (1980); and DeLuca et al., Arch. Biochem. Biophys., 225:285-291 (1983). Known

chemistries in the art may be adapted for use in attaching biomolecules to coated or uncoated silicon channel surfaces. A binding moiety such as an antigen binding protein, a polynucleotide probe, or one of a ligand/receptor pair may be attached to the silicon channel surfaces. The surface coated mesoscale flow systems can be utilized in any of a wide range of available binding assays known in the art such as immunoassays, enzymatic assays, ligand/binder assays, 5 polynucleotide hybridization assays, and cell surface binding assays. The detection of cellular or macromolecular analytes can be implemented by selecting the appropriate binding moiety coated on the surface of the detection region.

In addition, magnetic beads may be utilized in the device, which can be moved through the mesoscale flow system using an externally applied magnetic field, e.g., from a magnetic source located in an appliance utilized in combination with the device. The binding moiety or other reagent required in an assay may be immobilized on a magnetic bead to 10 enable, e.g., the delivery of the binding moiety to the detection region to bind to the analyte. After the binding of the analyte to the binding moiety attached to the magnetic bead, the analyte may be, e.g., further purified, or moved via an external magnetic field to a different detection region in the flow system for further analyses.

The binding of the analyte to the binding moiety in the detection region can be detected by any of a number of methods including monitoring the pressure or electrical conductivity of sample fluids in the device as disclosed herein or by 15 optical detection through a transparent cover either visually or by machine. Devices such as valves, mesoscale pressure sensors, and other mechanical sensors can be directly fabricated on the silicon substrate and can be mass-produced according to well established technologies. Angell et al., Scientific American 248:44-55 (1983).

The binding of an analyte to a binding moiety in the detection region can be detected optically. The simplest embodiment is one in which a positive result is indicated by an agglomeration or agglutination of particles, or development of 20 or change in color, which can be visually observed, optimally with the aid of a microscope. The optical detection of the binding of an analyte to a binding moiety in the mesoscale detection chambers can be implemented by the attachment of a detectable label, such as a fluorescent or luminescent molecule or polymeric support, such as a bead, to either the analyte or the binding moiety using assay protocols known per se. The luminescent or fluorescent label in the detection region can be detected by light microscopy through a translucent window disposed over the substrate. Analytes may be 25 detected by a luminescent or fluorescent signal produced by a binding moiety upon binding of the analyte. Alternatively, a second labelled substance, such as a fluorescent labelled antibody can be delivered through the flow system to bind to the bound analyte/binding moiety complex in the detection region to produce a "sandwich" including an optically detectable moiety whose presence is indicative of the presence of the analyte. For example, immunogold or immunofluorescent labels reported in the prior art may be utilized. (See, e.g., Rosenberg et al., Clin. Chem. 30: 1462-1466 (1984); 30 Rosenberg et al., Clin. Chem. 31: 1444-1448 (1985); and Goin et al., Clin. Chem. 32: 1655-1659 (1986)).

The binding of an analyte in a liquid biological fluid sample to a binding moiety in the detection region also may be detected by sensing electrical conductivity at some region within the device. The conductivity of liquid in the mesoscale flow paths can be measured in order to detect changes in electrical properties upon analyte binding to binding moieties 35 in the detection region. The conductivity may be measured, e.g., in the device 10 shown schematically in Figures 10 and 10b. Device 10 includes the silicon substrate 14 on which are microfabricated inlet ports 16 and flow channel 20. The substrate is covered by a translucent window 12. Electrical conductivity measurements are made using the electrical contacts 18 which are fabricated on the top side of the substrate in contact with the mesoscale sample flow channel 20, and which are connected to contacts 17 which extend through to the bottom of the substrate. The contacts 17 can be fabricated by known techniques of thermal gradient zone melting. (See Zemel et al., in: Fundamentals and Applications of Chemical Sensors, D. Schuetzle and R. Hammerle, Eds., ACS Symposium Series 309, Washington, DC, 1986, p. 2.) Device 10 may be nested in an appliance such as appliance 50, shown in Figure 4, capable of detecting conductivity changes through the contacts 17. Changes in conductivity can be correlated with changes in fluid properties, such 40 as fluid pressure, induced by analyte binding in the detection region.

The binding of an analyte to a binding moiety in the detection region also can be detected by monitoring the pressure 45 of the sample fluids in certain specially designed regions of the mesoscale flow passages. For example, a pressure detector connected to sample fluid entering and exiting the mesoscale flow system will allow the detection of pressure decreases caused by analyte binding and resulting clogging or flow restriction. Figure 4 shows schematically, as an example, device 10, which is nested within appliance 50, which includes two pressure detectors 54 for detecting flow 50 pressure of fluids entering and exiting device 10 through ports 16. When, during an assay, particles agglomerate or molecules chemically interact to form a network clogging the flow passage or increasing the viscosity of the liquid, that change can be detected as a pressure change indicative as a positive result. A mesoscale pressure sensor also may be fabricated directly on the silicon substrate. Angell et al., Scientific American 248: 44-55 (1983).

This form of detection of an analyte binding to a binding moiety in the detection region can be enhanced by 55 geometries sensitive to flow restriction in the flow system. In one embodiment, the mesoscale flow channels in the devices may be constructed with a "fractal" pattern, i.e., of a pattern of serially bifurcating flow channels. Figure 11 illustrates schematically one embodiment of a device 10 which includes substrate 14 microfabricated with two fractal flow systems 40. The fractally bifurcating channels may be fabricated on a silicon substrate with reduced dimensions at each bifurcation, providing sequentially narrower flow channels. the substrate. Fluid flow in these fractally constructed flow systems is very sensitive to fluid viscosity and to the development of flow restriction caused, for example, by the prolif-

eration of cells, or the agglomeration of cells, particles, or macromolecular complexes that may be present in a sample. The detection of the presence of an analyte based on flow restriction is described in WO-A-93/22054, published November 11, 1993.

The fractally designed microchannels readily allow, e.g., the growth of organisms in a culture to be monitored on the basis of flow impedance due to changes in fluid viscosity which can be detected, e.g., optically through a transparent cover over the substrate. The presence and growth of an organism in a sample will influence the flow characteristics within the fractal. One or more pressure sensors may be utilized to detect pressure changes due to changes in fluid properties caused by the presence of an analyte in or beyond the fractal flow paths. Changes in conductivity upon analyte binding also may be readily detected through electrical conductivity sensors in contact with the flow region. For example, clogging of the fractal region 40 of device 10, which blocks flow of analyte from input port 16A to outlet port 16B may be detected by a conventional conductivity probe 17, whose output is indicative of the presence or absence of aqueous fluid in the outflow channel. Binding moieties may be provided in fractal region, e.g., immobilized on the surface of the fractal flow path, or on a solid phase reactant such as a bead, to bind to the analyte and enhance flow restriction in the fractal flow path.

A large number of binding assay protocols known in the art may be exploited in the mesoscale detection systems of the invention.

The reaction of an analyte with a binding moiety in the detection region may be detected by means of an agglutination. A fluorescent or luminescent labelled molecule or bead capable of binding to the analyte or analyte/binding moiety complex in the detection region may be used to enable the detection of agglutination of the binding moiety and the analyte by light microscopy through a translucent cover over the detection region. For example, the agglutination of blood cells in a mesoscale detection chamber can serve as a positive test for the blood type of the sample. Antibodies may be coated, either chemically or by absorption, on the surface of the detection region to induce agglutination, giving a positive test for blood type. The blood sample may be mixed with a fluorescent dye to label the blood cells and to enable the optical detection of the agglutination reaction. Antibodies bound to fluorescent beads also may be utilized. A plurality of detection regions housing different antibodies may be fabricated in the mesoscale flow paths to allow the simultaneous assay of e.g., A, B and Rh blood types in one device.

Immunochemical assay techniques known in the art, such as antibody sandwich assays and enzyme-linked immunoassays, may be exploited in the mesoscale detection regions of the devices to detect a preselected analyte. (See Bolton et al., Handbook of Experimental Immunology, Weir, D.M., Ed., Blackwell Scientific Publications, Oxford, 1986, vol. 1, Chapter 26, for a general discussion on immunoassays.) In one embodiment, the analyte may be an antigen and the binding moiety may be a labelled antigen binding protein, e.g. a fluorescent labelled antibody. Alternatively a sandwich immunoassay can be performed wherein a tagged binding molecule, such as a fluorescent labelled antibody, is utilized to detectably bind to an analyte/binding moiety complex formed in the detection region. An example of a sandwich immunoassay is illustrated schematically in Figures 5A-D, wherein the surface of mesoscale flow channel 20 in substrate 14 is coated with an antibody 103 capable of binding an analyte 104. Figures 5B and 5C illustrate the binding of the analyte 104 to the antibody 103 in the flow channel. Bound analyte is then detected by the subsequent addition of a fluorescent labelled antibody 105 which complexes to the bound analyte as illustrated in Figure 5D. The fluorescent labelled complex can be detected through a translucent window over the detection region using a fluorometer.

Luminescence may be readily detected in the mesoscale flow systems of the devices, emitted from, e.g., a fluorescein labeled binding moiety. In one embodiment, luminescence emission may be readily detected in a mesoscale flow system, e.g., using a microplate reader, including a photomultiplier tube, or a camera luminometer. In one embodiment, the analyte may be detected by the use of a binding moiety comprising two antibodies capable of binding to the analyte, wherein one antibody is labeled with fluorescein, which emits light, and a second antibody is labeled with rhodamine, which absorbs light. When the rhodamine and fluorescein-labeled antibodies each bind to the analyte, a quenching of the fluorescein can be observed, indicating the presence of the analyte. Nakamura et al., eds., Immunochemical Assays and Biosensor Technology for the 1990s, American Society of Microbiology, Washington, DC, pp. 205-215. In one embodiment, the fluorescein labeled antibody is immobilized in the detection region. The analyte and the rhodamine-labeled antibody are then delivered to the detection region, and quenching of the fluorescein is observed indicating the presence of the analyte. In another embodiment, fluorescein-labeled antibodies conjugated to and coating a bacterial magnetic particle may be utilized in an immunoassay, wherein the antibody is capable of binding to the analyte. Nakamura et al. Anal. Chem. 63:268-272 (1991). In this embodiment, the agglutination of bacterial magnetic particles conjugated to the fluorescein-labeled antibody causes a fluorescence quenching, indicating a positive assay for the analyte. The agglutination and resulting quenching may be enhanced by applying a magnetic field to the mesoscale detection region, e.g., via a magnetic source located in an appliance used in combination with the appliance.

In another embodiment, polynucleotide hybridization assays known in the art may be performed (Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, 1989). As illustrated schematically in Figure 6, the surface of flow channel 20 in substrate 14 may be coated with a polynucleotide probe 110. Upon binding of the complementary analyte polynucleotide 104 to the immobilized polynucleotide probe 110, a second detectable, e.g., fluorescent labelled, macromolecular probe 105 can be added to bind to the sample polynucleotide. Detection of flu-

rescence indicates a positive assay.

In other embodiments, the mesoscale flow system may include a chamber for separating a selected cell population from a biological fluid sample in preparation for downstream analysis of either a macromolecule on or within the cells or of a component in the extracellular fluid. The mesoscale separating region includes immobilized binding moieties capable of reversibly binding a target cell via a characteristic cell surface molecule such as protein. The mesoscale dimension of the separation region kinetically enhances binding of the cell and the binding moiety. In one embodiment, the cells remain immobilized while extracellular fluid flows downstream and is analyzed. In another, flow may be continued to wash the cells, e.g., with a flow of buffer. At higher flow rates and pressures, the washed cells are released from the separation region and move downstream for analysis.

The devices of the invention also may include cell lysing means in fluid communication with the mesoscale flow channel to allow the cells to be lysed prior to analysis for an intracellular molecule such as an mRNA. As illustrated in Figure 8, the cell lysing means may comprise cell membrane piercing protrusions 124 extending from a surface of a flow channel 20. As fluid flow is forced through the piercing protrusion 124, cells are ruptured. Cell debris may be filtered off and intracellular analytes may then be analyzed. Sharp edged pieces of a material such as silicon also may be utilized, trapped with the mesoscale flow system to implement lysis of cells upon the applications of sufficient flow pressure. In another embodiment, the flow channel may simply comprise a region of restricted cross-sectional dimension which implements cell lysis upon application of sufficient flow pressure. These devices typically are used in connection with an appliance which includes means, such as a pump, for forcing the cell containing sample into the cell lysis means to cause cell lysis upon application of sufficient flow pressure. In addition, the cell lysis means may comprise a cell lysis agent. Cell lysing agents known in the art may be utilized.

As illustrated in Figure 7, the surface of a flow channel 20 may also include protrusions 122 constituting a cellular sieve for separating cells by size. As cell samples are flowed, typically under low pressure, through the flow channel, only cells capable of passing between the protrusions 122 are permitted to flow through in the flow channel.

The mesoscale devices also may be utilized to implement enzymatic reactions. Mesoscale enzyme reaction chambers fabricated in the substrate may be temperature controlled to provide optimal temperatures for enzyme reactions. Inlet ports may be provided, in fluid communication with the enzyme reaction chamber, to allow reagents and other required enzyme assay components to be added or removed. The assay devices embodying such chambers may be nested in an appliance such as appliance 50, illustrated schematically in Figure 4, having means to regulate the temperature of the enzyme reaction chambers and to deliver or recover assay components through flow channels 56 in appliance 50 and ports 16 in device 10. The appliance may be utilized to implement the timed addition of sample or reagent fluids to the devices. In order to regulate the temperature of the reaction chambers, the devices may be utilized in a nesting site in an appliance utilized in combination with the device. An electrical heating or cooling element may be provided in the nesting site for heating or cooling the reaction chamber in the device. Alternatively, electrical contacts may be provided in the substrate and may be mated with electrical contacts in the appliance to provide electrical resistance heating or cooling of the reaction chamber.

The polymerase chain reaction (PCR) may be performed in a mesoscale reaction chamber to enable the detection of a polynucleotide in a sample. Inlet ports in fluid communication with the reaction chambers permit addition of required reagents, such as nucleic acids, primers and Taq polymerase. The chain reaction may be performed, according to methods established in the art (Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989). Methods and apparatus for mesoscale PCR analyses are described in WO-A-93/22058, published November 11, 1993.

In another embodiment, the devices may be utilized to perform an enzyme reaction in which the mixing and addition of sample and reagent components is timed, as is illustrated in the device 10 shown schematically in Figure 21. The substrate 14 of device 10 is microfabricated with inlet ports 16, flow channels 20, the reaction chambers 22A and 22B and the detection chamber 22C. The reaction chambers 22A, and 22B each comprise a tortuous mesoscale flow channel. The path length of the tortuous channel can be designed to permit the timed mixing and addition of sample and reagent components. The device may be utilized in combination with an appliance with ports mated to ports in the device, capable of delivering and receiving fluids through the flow system of the device, and optionally, capable of optically detecting a positive result in the detection chamber. In one embodiment, the cholesterol content of a sample may be assayed. Cholesterol esterase is applied via inlet port 16A, and buffer and sample are added via inlet ports 16B and 16C. The mixture then flows through channel 20D to the tortuous mixing/reaction channel 22A. The time of mixing and reaction may be predetermined by microfabricating the tortuous channel with the appropriate length. Cholesterol oxidase is added via port 16D and flows through channel 20G to the tortuous channel 22B, where the timed mixing and reaction of the oxidase with the fluid from channel 22A occurs. A positive result can be detected optically by observing the detection chamber 22C through an optical window disposed over the substrate. The detection chamber 22C may be provided with a binding moiety capable of detectably reacting with the product of the enzyme reaction. The device may be applied to a range of clinical enzymatic and other reactions.

Optionally, depending on the protocol being exploited in the structure of the chip, the appliance may also be designed to inject reagents necessary to complete the assay, e.g., inject a binding protein tagged with an optically

detectable moiety, a substrate solution, or other reagents. The pressure of fluid flow in the mesoscale flow channel 20 in device 10 can be detected by the pressure detectors 54 provided in appliance 50. A microprocessor may be included in the appliance to assist in the collection of data for one or a series of assays. In order to enhance the accuracy of an assay, the substrate may be fabricated to include a control region in the flow system, e.g., a region which does not include binding moieties, such that the sample is directed to both the detection and control regions. Data obtained from sample fluid flowing through the control region may be detected and compared with the data from the sample detection region to increase the precision of the assay.

Example 1

A multitest device 10 including substrate 14, shown schematically in Figure 16, is used to detect the presence of an intracellular polynucleotide in a biological cell-containing fluid sample. The device is used in combination with an appliance, such as appliance 50, shown in Figure 5. The appliance includes fluid channels with ports, that include valves that may be reversibly opened and closed, mated to the ports in device 10, allowing the ports in the device to be mechanically opened and closed during an assay. The appliance also includes means, such as electrical contacts mated to contacts imbedded in the substrate 14, for regulating the temperature of reaction chambers 164 and 166. The appliance further includes a pump to control the flow of fluid through the device 10.

Initially, the valves in the appliance are used to close ports 16C and 16D, while ports 16A and 16B remain open. The sample is directed to the sample inlet port 16A by a pump in the appliance, and flows through the mesoscale flow path 20A to chamber 22A, which contains binding moieties immobilized on the wall of the chambers for selectively binding to a surface molecule on a desired cell population. After binding of the desired cell population in chamber 22A, flow with buffer is continued, exiting through port 16B, to purify and isolate the cell population. Port 16B is then closed and port 16C is opened. Flow is then increased sufficiently to dislodge the isolated cells from the surface of chamber 22A to chamber 22B where membrane piercing protrusions 124 in chamber 22B tear open the cells releasing intracellular material.

Sample flow continues past filter 168, which filters off large cellular membranes and other debris, to the mesoscale PCR chambers 164 and 166. The valves in the appliance are used to open port 16B and to close port 16A. Taq polymerase, primers and other reagents required for the PCR assay are added to chambers 164 and 166 through port 16C from a mated port and flow path in the appliance. A pump in the appliance connected via port 16B is used to cycle the PCR sample and reagents between chambers 164 and 166, set at 94°C and 65°C respectively, to implement a polynucleotide dehybridization and polymerization cycle, allowing the production and isolation of product polynucleotide. The valves in the appliance are used to close port 16C and to open port 16D. The pump in the appliance connected to port 16B is used to direct the polymerized polynucleotide isolated from the cell population to the fractal detection region 40, which contains immobilizing binding moieties, such as a complementary polynucleotide probe. Flow restriction in the fractal region 40 indicates a positive assay for the intracellular polynucleotide.

Example 2

A chemiluminescent peroxyoxylate organic phase reaction was conducted in a mesoscale flow channel. A Cyalume™ light stick (Aldrich, Milwaukee, WI) was opened and the mixture of peroxyoxylate and fluorophore (component A) were drained into a test tube. The glass vial containing the oxidant was removed and washed with alcohol. The contents of the vial (component B) were transferred to a test tube. A 100 µL sample of component A and 50 µL of component B were mixed together to initiate the chemiluminescent reaction.

A sample of the fluorescent solution was introduced into the central inlet port of chip #6, provided with a chamber with dimensions of 812 µm in width, 400 µm in depth and 5.2 mm in length, connected to two 20 µm deep, 100 µm wide, 3.25 mm long channels. Any excess sample was wiped off the surface of the chip, and the chip was placed into a modified microwell strip holder. The light emission from the mesoscale flow channel was measured using an Amerlite microplate reader (Amersham Diagnostics Ltd., Amersham, UK). A similar experiment was performed using a 300 µm wide, 20 µm deep mesoscale flow channel (volume 70.2 pL) in chip #5. Light emission (peroxyoxylate chemiluminescence) was detected and measured in units of RLU (relative light units) from the mesoscale flow channels in the different chips using the luminescence microplate reader (Table 1).

Table 1

| Chip | Channel Volume | Light Emission (RLU) |
|------|----------------|----------------------|
| #6 | 1702 pL | 718.26 |
| #5 | 70.2 pL | 35.63 |

Example 3

5 In an aqueous phase reaction, the chemiluminescent horseradish peroxidase catalyzed oxidation of isoluminol was examined. The luminol-hydrogen peroxide reagent was prepared as follows: Sodium luminol (12.5 mg) was dissolved in 50 mL of Tris buffer (0.1 mol/L, pH 8.6). 15.5 μ L of hydrogen peroxide (30% w/v) was mixed with 0.5 mL of Tris buffer (0.1 mol/L, pH 8.6). These two solutions were combined and protected from light. The luminol-hydrogen peroxide reagent (100 μ L), 5 μ L of 4-iodophenol ((Aldrich) (1 mg/ml in 0.1 mol/L Tris buffer, pH 8.6), and 10 μ L of a dilution of horse-
10 radish peroxidase (Type VIA, 1 mg/mL) in Tris buffer (0.1 mol/L, pH 8.6) were mixed together. A sample of this solution was introduced into the central chamber of chip #6 or into the 300 μ m channel of chip #5. The light emission was then measured using the Amerlite microplate reader.

10 The chemiluminescence emission from the horseradish peroxidase catalyzed oxidation of luminol in the different mesoscale channels was detected using the luminescence microplate reader. A peroxidase assay using dilutions of the
15 stock peroxidase gave a dose dependent relationship (Table 2).

Table 2

| Chip | Channel Volume | Peroxidase dilution | Light Emission (RLU) |
|------|----------------|---------------------|----------------------|
| #6 | 1702 pL | undiluted | 0.18* |
| | | 1:10 | 4.68 |
| | | 1:100 | 2.23 |
| | | 1:1000 | 1.82 |
| | | undiluted | 2.09 |
| #5 | 70.2 pL | | |

* Low light level because of substrate exhaustion.

Example 4

35 Chemiluminescent reactions in the mesoscale flow channels were detected photographically. Mesoscale channels of chip #6 were filled with the peroxyoxylate or horseradish peroxidase (10 μ g/mL)-luminol-peroxide reaction mixtures as described in Examples 2 and 3. The light emission was detected by contacting the chip with instant photographic film (Polaroid, Type 612) in a camera luminometer (Wolfson Applied Technology, Birmingham, UK). Light emission from the different chemiluminescent reactions in the mesoscale flow channels was detected using high speed instant photographic film (Table 3). The lower light intensity from the peroxidase reaction required a longer exposure time.
40

Table 3

| | Exposure Time | Detected (D) Not Detected (ND) |
|---------------------------------|---------------|--------------------------------|
| Peroxyoxylate reaction | 1 second | D |
| | 5 minutes* | D |
| Horseradish peroxidase reaction | 10 minutes | D |

* After 2 day incubation at room temperature.

55

Claims

1. A device (10) for detecting an analyte in a cell-containing fluid sample wherein the analyte is capable of specifically binding to an analyte-specific binding moiety, the device comprising:

a solid substrate (14) fabricated to define:

- a sample inlet port (16); and
- a mesoscale flow system (40) comprising:

5 a sample flow channel (20) extending from said inlet port; and
 an analyte detection region (22), in fluid communication with said flow channel (20), comprising said analyte-specific binding moiety for specifically binding said analyte, and at least a portion of said detection region (22) having a depth on the order of 0.1 to 500 μ m;
 10 a cell separation region (22A, 22B), upstream from said detection region (22), comprising a cell-specific binding moiety for binding a cell surface molecule of a cell population in said fluid sample, immobilised in said separation region;
 means for inducing flow of said sample to said separation region; and
 15 means for detecting the binding of the analyte to the analyte-specific moiety, thereby to determine the presence of the analyte.

2. The device of claim 1, wherein said analyte specific binding moiety is immobilised in said detection region (22).

3. The device of claim 1 or claim 2 further comprising:

20 a control region in fluid communication with said sample inlet port (16) and wherein said detecting means comprises:

25 a window for optically probing said detection region, disposed over said detection region (22) on said substrate (14); and
 a control region window, disposed over said control region on said substrate, for optically probing said control region.

30 4. The device of any one of claims 1 to 3, wherein the analyte is an intracellular molecular component in a cell-containing liquid biological sample, the device further comprising:

35 cell lysing means (124) in said mesoscale flow system (40) in fluid communication with said flow channel (20);
 and
 means for engaging said cells in said cell-containing sample within said substrate with said cell lysing means thereby to release said intracellular molecular component.

5. The device of any one of the preceding claims further comprising:

40 an appliance for use in combination with said substrate, said appliance comprising:

45 means for holding said substrate; and
 optical means for viewing the contents of said mesoscale flow system in said substrate.

6. The device of claim 5, wherein said optical means comprises magnifying optics and a video camera, and wherein said appliance further comprises:

50 a tilt mechanism for manually adjusting the angle and location of the device; and
 a video screen for viewing the contents of said flow system.

50 7. The device of any one of claim 1 to 4 further comprising:

an appliance for use with the analytical device, said appliance comprising:

55 means for holding said substrate;
 fluid input means interfitting with an inlet port on said substrate; and
 pump means for passing fluid through the flow system of said substrate when held in said holding means.

8. The device of claim 7, wherein said appliance further comprises:

a reservoir containing a labelled binding moiety capable of binding analyte in said sample; and means for delivering the labelled binding moiety to said flow system.

- 5 9. The device of any one of the preceding claims wherein said detection region (22) further comprises a tortuous mesoscale flow channel, microfabricated with a length allowing the timed mixing of fluid flowing through said tortuous channel.
- 10 10. The device of any one of the preceding claims, wherein the analyte-specific binding moiety is labelled.
11. A method for detecting the presence of an analyte in a fluid sample, the method comprising the steps of:
 - (i) providing the device (10) of any one of the preceding claims;
 - (ii) delivering a sample to said inlet port (16) and through said flow system (40) to said detection region (22) in said device; and
 - 15 (iii) detecting the binding of an analyte to said analyte-specific binding moiety in said detection region with said detecting means in said device, thereby to determine the presence of the analyte.

Patentansprüche

- 20 1. Vorrichtung (10) zum Detektieren eines Analyten in einer zellhaltigen Flüssigkeitsprobe, wobei der Analyt fähig ist, sich spezifisch an eine analytenspezifische Bindegruppe zu binden und wobei die Vorrichtung folgendes umfasst:
 - ein festes Substrat (14), das ausgebildet ist, folgendes zu definieren:
 - 25 eine Probeneinbringöffnung (16); und
 - ein Mesoscale-Strömungssystem (40), umfassend:
 - 30 einen Probenströmkanal (20), der sich von der Einbringöffnung weg erstreckt; und
 - einen Analytendetectionsbereich (22) in Flüssigkeitskommunikation mit dem Strömkanal (20), umfassend die analytenspezifische Bindegruppe zur spezifischen Bindung des Analyten und zumindest einen Abschnitt des Detektionsbereichs (22), der eine Tiefe in der Größenordnung von 0,1 bis 500 µm aufweist;
 - 35 einen Zellenabtrennbereich (22A, 22B) stromaufwärts vom Detektionsbereich (22), umfassend eine zellspezifische Bindegruppe zur Bindung eines Zelloberflächenmoleküls einer Zellpopulation in der Flüssigkeitsprobe, welche Bindegruppe im Abtrennbereich immobilisiert ist;
 - 40 ein Mittel, das das Strömen der Probe zum Abtrennbereich bewirkt; und
 - ein Mittel zum Detektieren der Bindung des Analyten an die analytenspezifische Gruppe, um dadurch das Vorhandensein des Analyten zu bestimmen.
- 45 2. Vorrichtung nach Anspruch 1, worin die analytenspezifische Bindegruppe im Detektionsbereich (22) immobilisiert ist.
3. Vorrichtung nach Anspruch 1 oder 2, weiters umfassend:
 - 50 einen Steuerbereich in Flüssigkeitskommunikation mit der Probeneinbringöffnung (16), worin das Detektionsmittel folgendes umfasst:
 - ein Fenster zum optischen Sondieren des Detektionsbereichs, das über dem Detektionsbereich (22) auf dem Substrat (14) angeordnet ist; und
 - 55 ein Steuerbereichsfenster, das über dem Steuerbereich auf dem Substrat angeordnet ist, um den Steuerbereich optisch zu sondieren.
4. Vorrichtung nach einem der Ansprüche 1 bis 3, worin der Analyt eine intrazelluläre molekulare Komponente in einer

zellhaltigen, flüssigen, biologischen Probe ist, wobei die Vorrichtung weiters umfasst:

ein Zellysemittel (124) im Mesoscale-Strömungssystem (40) in Flüssigkeitskommunikation mit dem Strömkanal (20); und

5 ein Mittel zum Zusammenbringen der Zellen in der zellhaltigen Probe innerhalb des das Zellysemittel enthaltenden Substrats, um dadurch die intrazelluläre molekulare Komponente freizusetzen.

5. Vorrichtung nach einem der vorhergehenden Ansprüche, weiters umfassend:

10 ein Gerät zur Verwendung in Kombination mit dem Substrat, wobei das Gerät folgendes umfasst:

ein Mittel zum Halten des Substrats; und

15 ein optisches Mittel zum Beobachten des Inhalts des Mesoscale-Strömungssystems im Substrat.

6. Vorrichtung nach Anspruch 5, worin das optische Mittel eine Vergrößerungsoptik und eine Videokamera umfasst und das Gerät weiters folgendes umfasst:

20 einen Neigungsmechanismus zum händischen Einstellen des Winkels und Standorts der Vorrichtung; und

einen Bildschirm zum Beobachten des Inhalts des Strömungssystems.

7. Vorrichtung nach einem der Ansprüche 1 bis 4, weiters umfassend:

25 ein Gerät zur Verwendung mit der analytischen Vorrichtung, umfassend:

ein Mittel zum Halten des Substrats;

30 ein Flüssigkeitszufuhrmittel, das mit einer Einbringöffnung auf dem Substrat zusammenpasst; und

ein Pumpenmittel, um Flüssigkeit durch das Strömungssystem des Substrats hindurchzuschicken, wenn es im Halterungsmittel gehalten wird.

35 8. Vorrichtung nach Anspruch 7, worin das Gerät weiters folgendes umfasst:

einen Speicherbehälter, der eine markierte Bindegruppe enthält, die Analyten in der Probe binden kann; und

ein Mittel zur Zufuhr der markierten Bindegruppe zum Strömungssystem.

40 9. Vorrichtung nach einem der vorhergehenden Ansprüche, worin der Detektionsbereich (22) weiters einen gewundenen Mesoscale-Strömkanal umfasst, der mit einer Länge mikrofabriziert ist, die das zeitlich abgestimmte Vermischen der durch den gewundenen Kanal strömenden Flüssigkeit ermöglicht.

45 10. Vorrichtung nach einem der vorhergehenden Ansprüche, worin die analytenspezifische Bindegruppe markiert ist.

11. Verfahren zum Detektieren des Vorhandenseins eines Analyten in einer Flüssigkeitsprobe, wobei das Verfahren die folgenden Schritte umfasst:

50 (i) Bereitstellen der Vorrichtung (10) nach einem der vorhergehenden Ansprüche;

(ii) Zuführen einer Probe zur Einbringöffnung (16) und durch das Strömungssystem (40) zum Detektionsbereich (22) in der Vorrichtung; und

55 (iii) Detektieren der Bindung eines Analyten an die analytenspezifische Bindegruppe im Detektionsbereich mit dem Detektiertmittel im Gerät, um dadurch das Vorhandensein des Analyten zu bestimmen.

Revendications

1. Dispositif (10) pour détecter un analyte dans un échantillon de fluide contenant des cellules où l'analyte est capable de se lier spécifiquement à une entité de liaison spécifique de l'analyte, le dispositif comprenant :

5 un substrat solide (14) usiné pour définir :

10 un orifice d'entrée de l'échantillon (16); et

15 un système d'écoulement à l'échelle méso (40) comprenant :

20 un canal d'écoulement de l'échantillon (20) s'étendant à partir dudit orifice d'entrée; et

25 une région de détection de l'analyte (22) en communication de fluide avec ledit canal d'écoulement (20) comprenant ladite entité de liaison spécifique de l'analyte pour lier spécifiquement ledit analyte et au moins une portion de ladite région de détection (22) ayant une profondeur de l'ordre de 0,1 à 500 µm;

30 une région de séparation des cellules (22A, 22B) en amont de ladite région de détection (22), comprenant une entité de liaison spécifique des cellules pour lier une molécule de surface de cellule d'une population de cellules dans ledit échantillon de fluide, immobilisée dans ladite région de séparation;

35 un moyen pour induire l'écoulement dudit échantillon vers ladite région de séparation; et

40 un moyen pour détecter la liaison de l'analyte à l'entité spécifique de l'analyte pour ainsi déterminer la présence de l'analyte;

2. Dispositif de la revendication 1, où ladite entité de liaison spécifique de l'analyte est immobilisée dans ladite région de détection (22).

35 3. Dispositif de la revendication 1 ou de la revendication 2, comprenant de plus :

40 une région de contrôle en communication de fluide avec ledit orifice d'entrée de l'échantillon (16) et où ledit moyen de détection comprend :

45 une fenêtre pour sonder optiquement ladite région de détection, disposée au-dessus de ladite région de détection (22) sur ledit substrat (14); et

50 une fenêtre de région de contrôle disposée au-dessus de ladite région de contrôle sur ledit substrat pour sonder optiquement ladite région de contrôle.

4. Dispositif selon l'une quelconque des revendications 1 à 3, où l'analyte est un composant moléculaire intracellulaire dans un échantillon biologique liquide contenant des cellules, le dispositif comprenant de plus :

45 un moyen de lyse des cellules (124) dans ledit système d'écoulement (40) à l'échelle méso en communication de fluide avec ledit canal d'écoulement (20); et

50 un moyen pour engager lesdites cellules dans ledit échantillon contenant des cellules dans ledit substrat avec ledit moyen de lyse des cellules pour ainsi libérer ledit composant moléculaire intracellulaire.

5. Dispositif selon l'une quelconque des revendications précédentes comprenant de plus :

55 un appareil à utiliser en combinaison avec ledit substrat, ledit appareil comprenant :

60 un moyen pour maintenir ledit substrat; et

65 un moyen optique pour voir les contenus dudit système d'écoulement à l'échelle méso dans ledit substrat.

6. Dispositif de la revendication 5, où ledit moyen optique comprend de l'optique d'agrandissement et une caméra

vidéo et où ledit appareil comprend de plus :

un mécanisme de basculement pour ajuster manuellement l'angle et l'emplacement du dispositif; et

5 un écran vidéo pour voir les contenus dudit système d'écoulement.

7. Dispositif selon l'une quelconque des revendications 1 à 4, comprenant de plus :

10 un appareil à utiliser avec le dispositif analytique, ledit appareil comprenant :

15 un moyen pour maintenir ledit substrat;

un moyen d'entrée de fluide collaborant avec un orifice d'entrée sur ledit substrat; et

15 un moyen formant pompe pour faire passer le fluide à travers le système d'écoulement dudit substrat quand il est maintenu dans ledit moyen de maintien.

8. Dispositif de la revendication 7, où ledit appareil comprend de plus :

20 un réservoir contenant une entité de liaison marquée capable de lier l'analyte dans ledit échantillon; et

un moyen pour délivrer l'entité de liaison marquée audit système d'écoulement.

9. Dispositif selon l'une quelconque des revendications précédentes où ladite région de détection (22) comprend de 25 plus un canal d'écoulement tortueux à l'échelle méso, micro-usiné à une longueur permettant le mélange temporelisé du fluide s'écoulant à travers ledit canal tortueux.

10. Dispositif selon l'une quelconque des revendications précédentes, où l'entité de liaison spécifique de l'analyte est marquée.

30 11. Méthode pour détecter la présence d'un analyte dans un échantillon de fluide, la méthode comprenant les étapes de :

(i) prévoir le dispositif (10) selon l'une quelconque des revendications précédentes;

35 (ii) délivrer un échantillon audit orifice d'entrée (16) et par ledit système d'écoulement (40) à ladite région de détection (22) dans ledit dispositif; et

détection avec ledit moyen de détection dans ledit dispositif pour ainsi déterminer la présence de l'analyte.

40

45

50

FIG.1

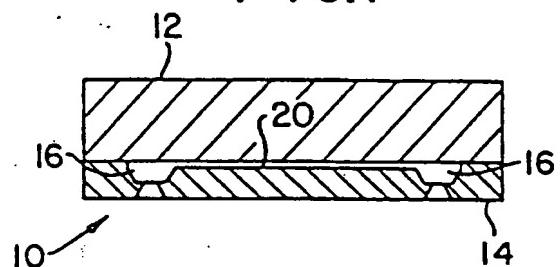


FIG.2

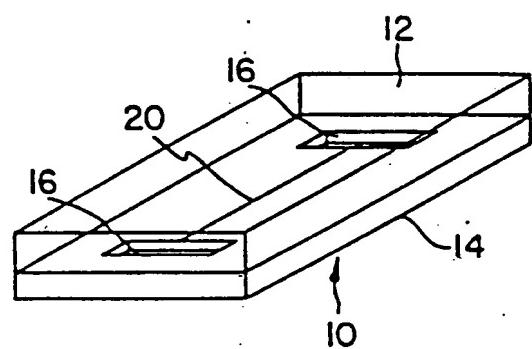


FIG.3

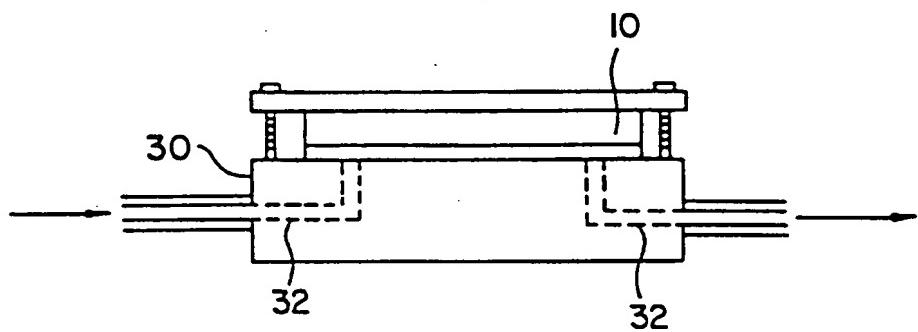


FIG. 4

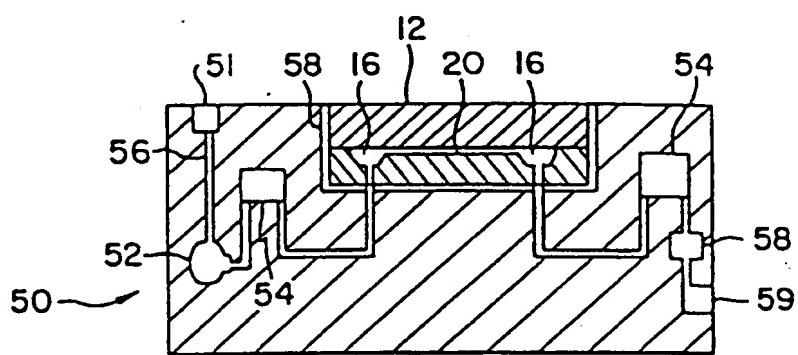


FIG. 5 A

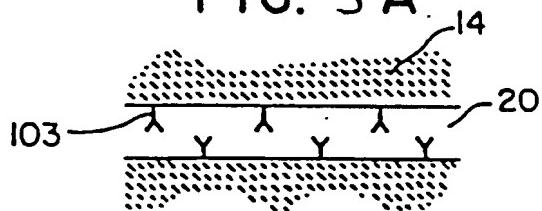


FIG. 5 B

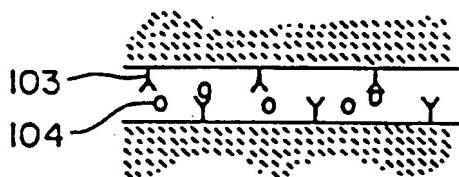


FIG. 5 C

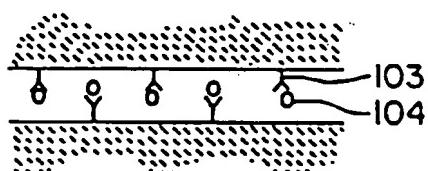


FIG. 5 D

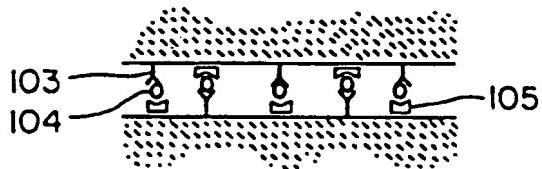


FIG. 6A

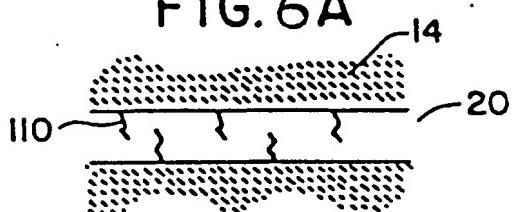


FIG. 6B

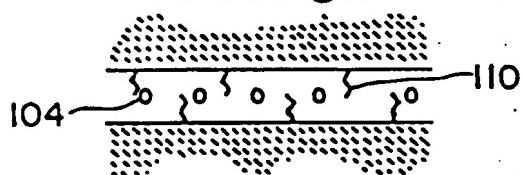


FIG. 6C

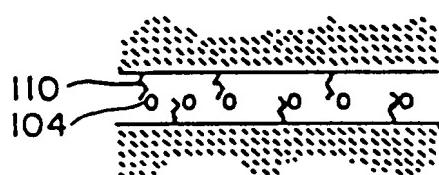


FIG. 6D

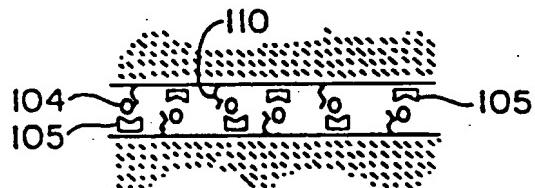


FIG. 7

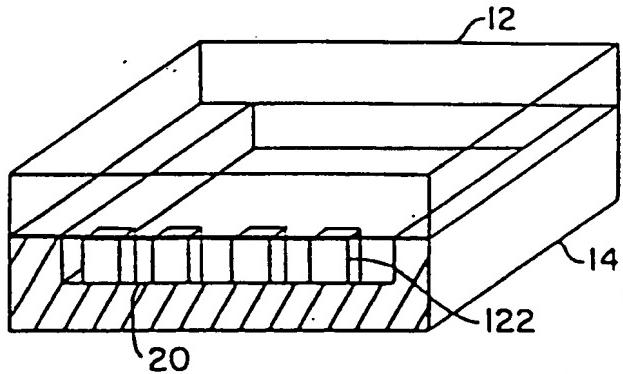


FIG. 8

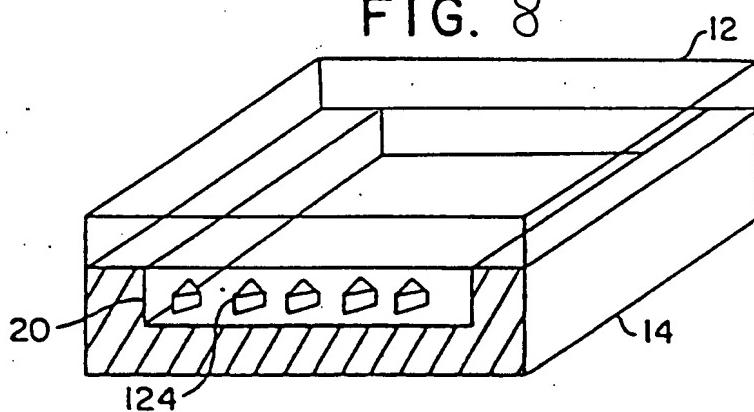


FIG. 9

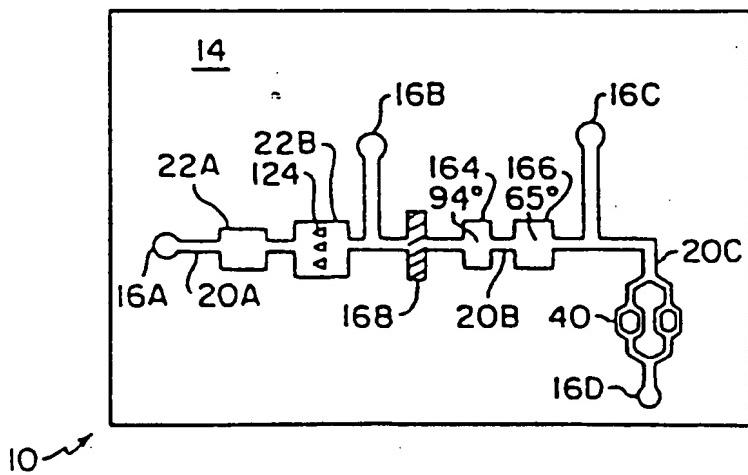


FIG. 10b

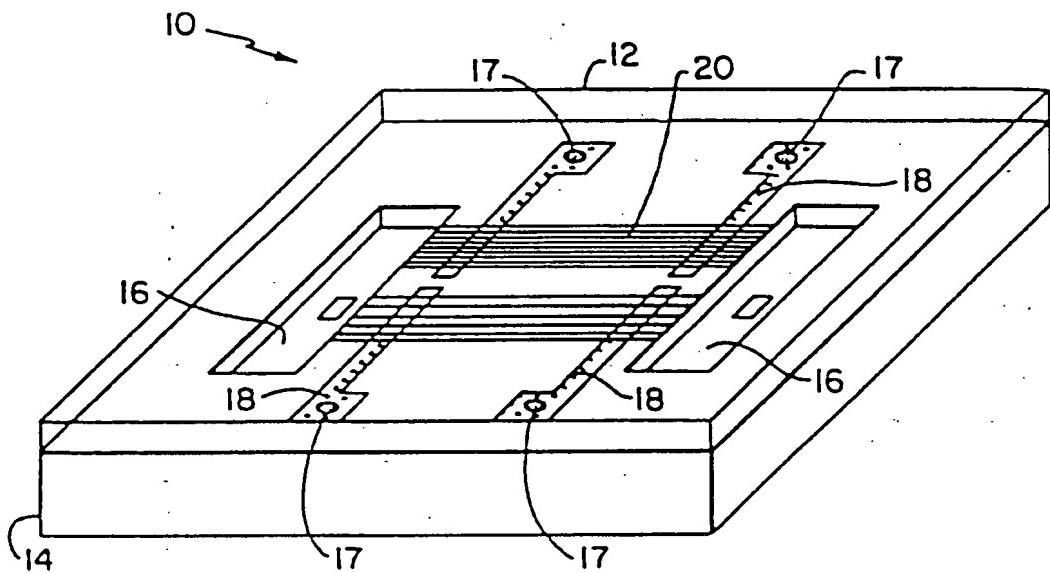


FIG. 10a

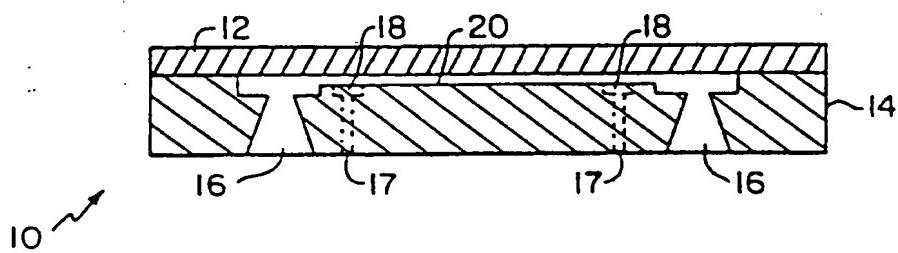


FIG. 11

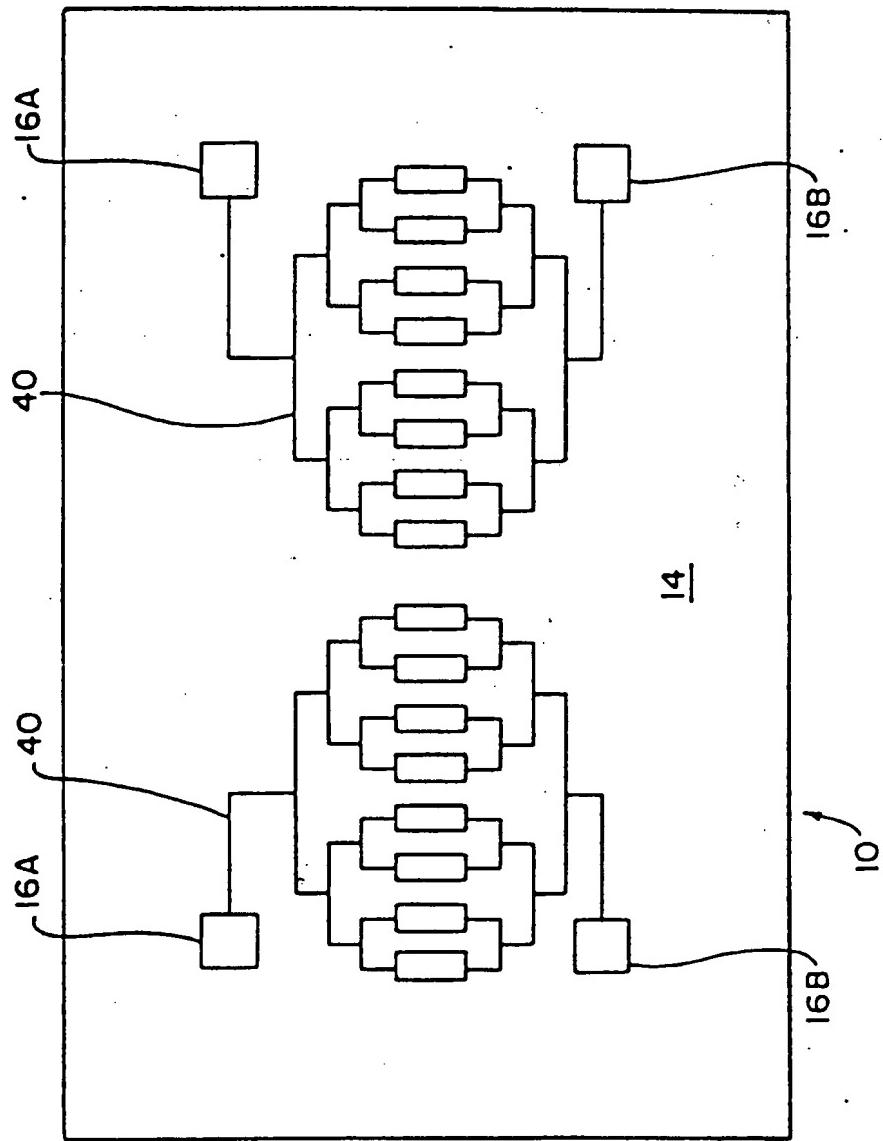


FIG. 13

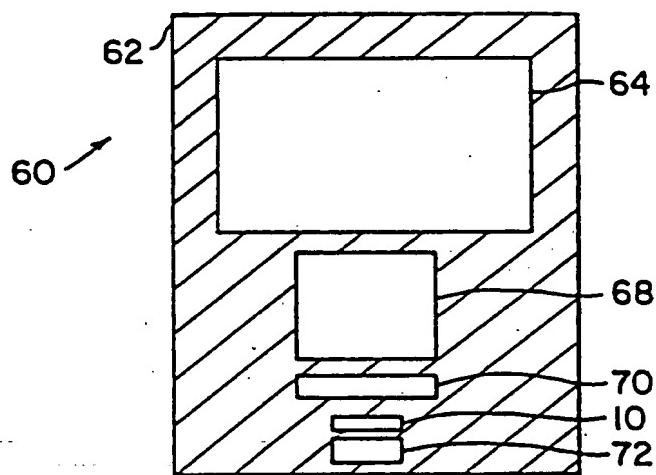


FIG. 12

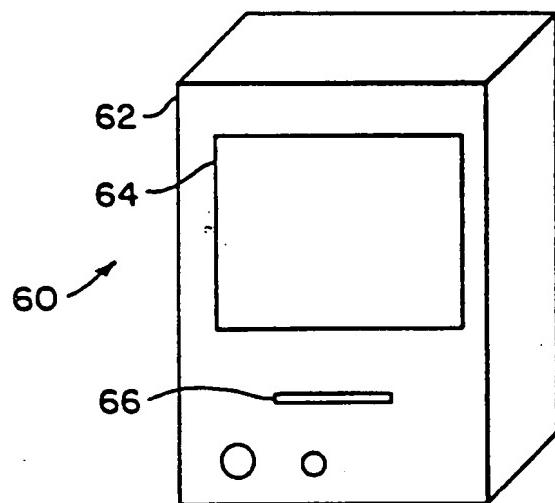
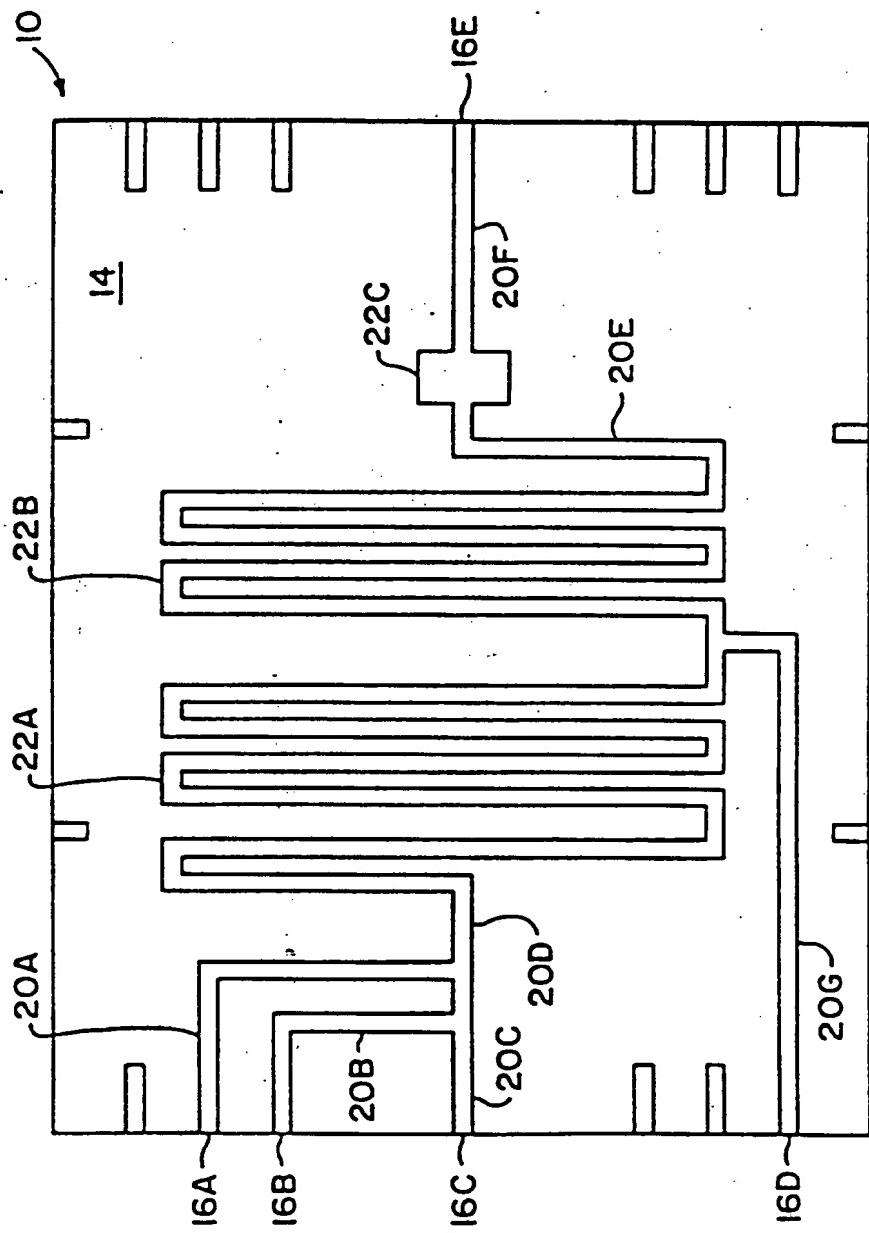


FIG. 14



(19)



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(54) Cell potential measurement apparatus having a plurality of microelectrodes

Zellpotentialmessvorrichtung mit mehreren Mikroelektroden

Appareil de mesure du potentiel cellulaire avec plusieurs microélectrodes

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EP 0 689 051 B1

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description

[0001] This invention relates to a cell potential measurement apparatus which is used in the field of electro-neurophysiology for measuring the potential change associated with activities of nerve cells or nerve organs.

[0002] Recently, medical investigations into nerve cells and the possibility of using nerve cells as electric elements have been actively pursued. When nerve cells are active, an action potential is generated. This action potential arises from a change in ion concentration inside and outside the cell membrane which is accompanied by a change in ion permeability in nerve cells and thus from the change in cell membrane potential accompanied thereby. Therefore, measuring this potential change accompanied by the ion concentration change (that is, the ion current) near the nerve cells with electrodes enables the detection of activities of nerve cells or nerve organs.

[0003] In order to measure the above-mentioned potential arising from cell activities, it is possible, for example, to insert an electrode comprising glass into an area of cells to measure the extracellular potential. When the evoked potential due to stimulation is measured, a metal electrode for stimulation is inserted together with a glass electrode for recording. However, measurement by the insertion of these electrodes has the possibility of damaging the cells, and measurement over a long period of time is difficult to carry out. In addition, due to restrictions of space and the need for operating accuracy, multi-point simultaneous measurements are also difficult to carry out.

[0004] Therefore, the present inventors developed a planar electrode comprising an insulation substrate and a multiplicity of microelectrodes, their conductive patterns being formed thereon with the use of a conductive material, and a cell culture could be placed on that surface (disclosed in Laid-open Japanese patent application Nos. (Tokkai Hei) 6-78889 and 6-296595). With this planar electrode, multi-point simultaneous measurements of potential change can be carried out without being affected by restrictions of space at a plurality of points with a short electrode-to-electrode distance. Also, this electrode enables long-term measurement.

[0005] An apparatus for cell potential measurement is described in BIOSENSORS & BIOELECTRONICS, vol. 9, no. 1994, 1994, AMSTERDAM NL, pages 737-741, W. NISCH ET AL.: 'A thin film microelectrode array for monitoring extracellular neuronal activity in vitro'. This apparatus uses a microelectrode array being provided on a glass substrate. By using this apparatus neuronal electrical activity can be recorded and analyzed.

[0006] In EP-0 585 933 A2 a planar electrode has been described. This planar electrode enables multi-point simultaneous stimulation and measurement of nerve cells as well as signal transmission and observation throughout many cells.

[0007] However, a measurement apparatus which

can efficiently use this kind of planar electrode, conduct measurements accurately and efficiently, and improve the measurement results has been strongly desired. Therefore, it is an object of this invention to provide a cell potential measurement apparatus which is capable of accomplishing these needs in the art.

[0008] In order to accomplish these and other objects and advantages, a cell potential measurement apparatus of this invention comprises the features of claim 1.

[0009] It is preferable that the cell potential measurement apparatus of this invention further comprises an optical observation means for observing the cells optically. It is also preferable that the cell potential measurement apparatus of this invention further comprises a cell culturing means for maintaining an environment for culturing cells which are placed on the integrated cell holding instrument. This configuration enables measurement over a long period of time.

[0010] Generally, the measurement conducted by means of the above-configured apparatus of this invention is carried out, for example, in the following steps. Sample cells are placed in a cell holding part of an integrated cell holding instrument, and a plurality of microelectrodes contact the cells.

[0011] An image of the cells is obtained by an optical observation means. A stimulation signal is applied between a pair of electrodes selected optionally from the plurality of microelectrodes by a stimulation signal supply means via an electric connection means. A change of the evoked potential over time which is obtained in each of the other electrodes is provided to a signal processing means via the electric connection means, which is then output, for example, to a display device etc. after going through the necessary signal processing. The measurement of the spontaneous potential which is not provided with a stimulation signal is carried out in a similar way.

[0012] The above-mentioned electro-chemical measurement of cells must be conducted in a condition in which the cells are alive. Therefore, it is common to use cultured cells, and the cell holding part of the integrated cell holding instrument can be equipped with a culture medium. Since the integrated cell holding instrument is detachable from the measurement apparatus, each integrated cell holding instrument can be placed inside an ordinary incubator for cell cultures and then taken out from the incubator and placed in the measurement apparatus. When a cell culturing means is further provided to maintain an environment for culturing the cells on the integrated cell holding instrument, long-term measurement is enabled. This cell culturing means comprises a temperature adjustment means for maintaining a constant temperature, a means for circulating a culture solution, and a means for supplying a mixed gas of air and carbon dioxide (e.g., CO₂ 5%).

[0013] It is preferable that the integrated cell holding instrument comprises a plurality of microelectrodes arranged in a matrix form (latticed) on the surface of a

glass plate, conductive patterns for drawing these microelectrodes, electric contact points which are connected to edge parts of these conductive patterns, and a coating of insulation covering the surface of these conductive patterns, and the cell holding part is disposed in an area including the plurality of microelectrodes.

[0014] The use of a transparent glass plate as the substrate facilitates optical observations of the cells. Therefore, it is preferable that the conductive patterns or the insulation coating are also substantially transparent or translucent. Furthermore, when the plurality of microelectrodes is arranged in a matrix form, it is easier to specify positions of electrodes which are applied with stimulation signals or electrodes where voltage signals arising from cell activities are detected. For example, it is preferable to arrange 64 microelectrodes in 8 columns and 8 rows. In addition, the surface area of each electrode should be as great as possible for reducing surface resistance and enhancing detection sensitivity. However, taking restrictions etc. arising from the electrode-to-electrode distance and space resolution of measurement into consideration, it is preferable that each electrode has a surface area of from $4 \times 10^2 \mu\text{m}^2$ to $4 \times 10^4 \mu\text{m}^2$.

[0015] According to the configuration of the invention, fixation of the glass plate and routing the microelectrodes to the outside can be performed easily and accurately. Furthermore, it is preferable that the electric connection means not only fixes the holder, but also comprises a printed circuit board having an outside connection pattern which is connected to the contact of the holder via a connector. As a result, connection with outside instruments, namely, with a stimulation signal supply means and a signal processing means is facilitated. For the transmission of stimulation signals or detection signals with as little attenuation and distortion as possible, the contact resistance of the electric contact point with the contact as well as the contact resistance of the contact with the connector are both preferably below 30 m ohm.

[0016] In addition, it is preferable that the optical observation means comprises an optical microscope, and an image pick-up device and an image display device connected to the optical microscope. In other words, the image of the cells which is enlarged by a microscope is picked up by an image pick-up device (e.g., video camera) and then displayed in an image display device (e.g., a high-accuracy display), so that it is easier to conduct the measurement while observing the cells and the electrode position. More preferably, when the optical observation means is further comprised of an image storage device, it is possible to record measurement results.

[0017] Also, when a pulse signal generator is used as the stimulation signal supply means, various kinds of signal waveforms can be applied as stimulation signals to the cells. It is preferable that the signal processing means comprises a multi-channel amplifier which amplifies a detection signal arising from cell activities and

a multi-channel display device which displays an amplified signal waveform in real-time, and that signal waveforms (change of cell potential over time) obtained from a plurality of electrodes can be displayed simultaneously.

- 5 [0018] It is preferable that a computer is provided to output the stimulation signal via a D/A converter, and at the same time, to receive and process an output signal arising from electric physiological activities of the cells via an A/D converter. As a result, the stimulation signal can be determined as an optional waveform on the screen or a waveform of a detection signal can be displayed on the screen. In addition to these operations, it is easier to display these signals after being processed in various forms or to output them to a plotter or to store them. Furthermore, with the use of this computer, the optical observation means and the cell culturing means can be controlled.
- 10 [0019] FIG. 1 is a perspective view showing an integrated cell holding instrument used for a cell potential measurement apparatus in one embodiment of this invention.
- 15 [0020] FIG. 2 is an assembly diagram of an integrated cell holding instrument.
- 20 [0021] FIG. 3 is a top view showing 64 microelectrodes and conductive patterns disposed in the center of a planar electrode comprising an integrated cell holding instrument.
- 25 [0022] FIG. 4 is a model cross-sectional view of a planar electrode.
- 30 [0023] FIGS. 5 (A) and 5 (B) are a top view and a side cross-sectional view showing a state in which a planar electrode is fixed by being held between upper and lower holders.
- 35 [0024] FIG. 6 is a perspective view of the planar electrode and the upper and lower holders of FIGS. 5 (A) and 5 (B).
- 40 [0025] FIG. 7 is a side view of a contact attached to an upper holder.
- 45 [0026] FIG. 8 is an assembly diagram of an integrated cell holding instrument seen from an opposite direction of FIG. 2.
- 50 [0027] FIG. 9 is a block diagram of a cell potential measurement apparatus in one embodiment of this invention.
- 55 [0028] FIGS. 10 (A) and 10 (B) are graphs showing one comparative example of a voltage waveform arising from activities of cultured cells measured by means of an integrated cell holding instrument used in this invention and a voltage waveform measured by means of a conventional general purpose glass electrode (electrode for measurement of extracellular-potential).
- [0029] FIGS. 11 (A) to 11 (C) are diagrams showing measurement results of the spontaneous potential of cultured cells measured by using an apparatus of this invention.
- [0030] FIGS. 12 (A) to 12 (C) are diagrams showing measurement results of the evoked potential of cultured

cells measured by using an apparatus of this invention. [0031] This invention will now be described in detail by referring to the attached figures and the following examples,. The examples are illustrative and should not be construed as limiting the invention in any way.

[0032] First, an integrated cell holding instrument used for a cell potential measurement apparatus of this invention will be explained. The integrated cell holding instrument 1, as shown as a perspective view in FIG. 1 and as an assembly diagram in FIG. 2, comprises a planar electrode 2, which is disposed with a plurality of microelectrodes and their conductive patterns on the surface of a glass plate, half-split holders 3, 4 for fixing the planar electrode 2 by holding it from the top and bottom, and a printed circuit board 5 on which these holders are fixed.

[0033] The planar electrode 2 is approximately the same as that disclosed in Laid-open Japanese patent application No. (Tokkai Hei) 6-78889 and others. The planar electrode 2 comprises, for example, a substrate made of a transparent plexiglass having a thickness of 1.1 mm and a size of 50 x 50 mm, and in the center of this substrate, 64 microelectrodes 11 are formed in a matrix form of 8 x 8, and each microelectrode is connected to a conductive pattern 12 (cf. FIG. 3). Each of the electrodes 11 has a size of 50 x 50 μm square (area $25 \times 10^2 \mu\text{m}^2$), and the center-to-center distance between adjacent electrodes is 150 μm . Furthermore, each side of the substrate has 16 electric contact points 7 totalling to 64 points (cf. FIG. 2). These electric contact points 7 are connected with the 64 microelectrodes 11 disposed in the center of the substrate to correspond 1 to 1 to the conductive patterns 12. The 16 electric contact points 7 on each side are arranged with a pitch of 1.27 mm. Next, a method of manufacturing this planar electrode 2 will be explained based on its cross-sectional view shown as FIG. 4. Each part in FIG. 4 is shown in an enlarged scale for convenience.

[0034] ITO (indium tin oxide), for example, is applied to form a layer of 150 nm thickness on the surface of a glass plate 13, which is then formed into the conductive patterns 12 using a photoresist and etching. On top of this layer, a negative photosensitive polyimide is applied to form a layer of 1.4 μm thickness, which is then formed into an insulation film 14 in a similar manner. The ITO layer is exposed at the microelectrodes, and at the electric contact points, and nickel 15 of 500 nm thickness and gold 16 of 50 nm thickness, are coated on these parts. A cylindrical polystyrene frame 6 (cf. FIG. 2) with an inner diameter of 22 mm, an outer diameter of 26 mm, and a height of 8 mm is adhered (via a conductive pattern 8 and an insulation-film 9) on the glass plate 13 using a silicone adhesive. This cylindrical polystyrene frame 6 is fixed with its center matching the center of the glass plate 13, that is, the central part of the 64 microelectrodes, and the inside of the polystyrene frame 6 becomes a cell holding part. The inside of this polystyrene frame 6 is filled with a solution comprising 1 wt.

% of chloroplatinic acid, 0.01 wt.% of lead acetate, and 0.0025 wt.% of hydrochloric acid. An electric current of 20 mA/cm² is generated for 1 minute to deposit platinum black 11a on the surface of the gold plating of the microelectrodes.

[0035] Next, the half-split holders 3, 4 for fixing the planar electrode 2 by holding from the top and bottom will be explained. The holders 3, 4 made, for example, of resin are provided with a stage part for holding a frame part of the planar electrode 2 and with a rectangular opening in the central part, as shown in FIG. 2. The upper holder 3 is equipped with a pair of fixtures 8 and 16 x 4 pairs of contacts 9.

[0036] A top view of the holders 3, 4 which hold and fix the planar electrode 2 is shown in FIG. 5 (A), and its side view (B-B cross-sectional view) is shown in FIG. 5 (B), and its perspective view seen from a bottom side is shown in FIG. 6. As clearly shown in these figures, the fixture 8 is pivoted on two opposing sides of the upper holder 3 by an axis pin 8a. Furthermore, a groove 4a is formed on two opposing sides of the lower holder 4 in the bottom face. By fitting a convex part 8b of the fixture 8 into the groove 4a, the upper and the lower holders 3, 4 are firmly fixed with the planar electrode 2 held in between.

[0037] The 64 contacts 9, which are disposed in the upper holder 3 to correspond to the electric contact points 7 of the planar electrode 2, are formed by processing an elastic, conducting metal plate such as a plate comprising BeCu coated with Ni and Au, and the contact 9 has a shape shown in FIG. 7. In other words, the contact 9 is comprised of a pin part 9a, and its base part 9b, and a movable contact part 9d extending from the base part 9b via a curved part 9c. According to this structure, the movable contact part 9d is capable of elastic displacement against the base part 9b. The upper holder 3 has 64 (16 x 4) holes which are inserted with the pin parts 9a of the contacts 9, and the same number of grooves are also formed which fit the base parts 9b.

[0038] As shown in FIG. 2 and FIG. 5 (B), the pin part 9a protrudes from the upper holder 3 at the point where the contact 9 is inserted into the above-mentioned hole and the groove and fixed. By alternately arranging the contacts 9 having two different lengths of the base part 9b, 16 pin parts 9a protruding from the upper holder 3 are lined in two staggered rows. This pin part 9a is connected to a connector which is mounted on a printed circuit board 5 used for connection with the outside.

[0039] On the other hand, the movable contact part 9d of the contact 9 protrudes from the bottom face of the upper holder 3 at the point where the contact 9 is inserted into the holder and the groove of the upper holder 3 and fixed. This arrangement is shown in FIG. 8, which is an assembly diagram seen from the side opposite the assembly diagram of FIG. 2. In this state, the planar electrode 2 is fixed between the holders 3, 4, the movable contact part 9d of each contact 9 touches the electric contact point 7 of the planar electrode 2, and a pre-

determined contact pressure is exerted on the contact part due to elastic deformation of the curved part 9c. In this way, the electric contact point 7, which is connected to the microelectrode 11 of the planar electrode 2 by way of the conductive pattern 12, is electrically connected with small contact resistance (less than 30 m ohm) to the contact 9.

[0040] Next, the printed circuit board 5 will be explained. This printed circuit board 5 serves not only for fixing the assembly of the planar electrode 2 and the holders 3, 4, but also for routing an electrical connection via a connector to the outside, starting from the microelectrode 11 of the planar electrode 2 via the conductive pattern 12 via the electric contact point 7 to the contact 9. Furthermore, this printed circuit board 5 facilitates handling procedures, for example, installation to the measurement apparatus.

[0041] This printed circuit board 5 comprises a glass epoxy substrate disposed with double-faced patterns, and on the back face shown in FIG. 8, a connector 5a is disposed at four parts surrounding a circular opening formed in the center. By inserting the 16 pin parts 9a which are protruding in two staggered rows from the four surface parts of the upper holder 3 into each corresponding connector 5a, the assembly of the planar electrode 2 and the holders 3, 4 is fixed at the printed circuit board 5, and at the same time, they are connected electrically.

[0042] At an edge part 5b on both sides of the printed circuit board 5, electric contact points are formed at 2.54 mm pitch used for a double-faced connector edge, and these electric contact points and the connectors 5a in the central part are connected by conductive patterns 5c. An inner row of the double-sided connector 5a is formed by a surface pattern, whereas an outer row is formed by a back side pattern, and each of the edge parts 5b is provided with 32 electric contact points formed for both sides together, totalling 64 electric contact points. For the purpose of assuring mechanical fixation, the upper holder 3 can be fixed to the printed circuit board 5 using a vise.

[0043] A preferable configuration of a cell potential measurement apparatus using the above-configured integrated cell holding instrument 1 is shown in FIG. 9. The measurement apparatus of this embodiment comprises the above-mentioned integrated cell holding instrument 1, an optical observation means 20 including an inverted microscope 21 for optical observations of cells which are placed in this integrated cell holding instrument 1, a computer 30 including a means of providing a stimulation signal to the cells and a means of processing an output-signal from the cells, and a cell culturing means 40 for maintaining a suitable culture medium for the cells.

[0044] Besides the inverted microscope 21 (for example, "IMT-2-F" or "IX70" manufactured by OLYMPUS OPTICAL CO., LTD.) where the integrated cell holding instrument 1 is installed, the optical observation means

- 20 also includes a SIT camera 22 used for a microscope (for example, "C2400-08" manufactured by HAMAMATSU PHOTONICS K.K.), a high-accurate display 23, and an image filing device 24 (for example, "TQ-2600" or "FTQ-3100" manufactured by MATSUSHITA ELECTRIC INDUSTRIAL CO., LTD.). A SIT camera is a general term used for cameras which apply a static induction transistor to an image pickup tube, a SIT camera being a representative example of sensitive cameras.
- 5 10 15 20 25 30 35 40 45 50 55
- However, the high-accuracy display 23 can be used also as a display for the computer 30. The specific devices described above in parenthesis are illustrative examples, and the invention is not limited to these devices only. This is also true for the examples shown in the following.
- [0045] As for the computer 30, a personal computer (for example, compatible with WINDOWS) is used which is mounted with an A/D conversion board and software for measurement. The A/D conversion board includes an A/D converter 31 and a D/A converter 32 shown in FIG. 9. The A/D converter 31 has 16 bits and 64 channels, and the D/A converter 32 has 16 bits and 8 channels.
- [0046] The measuring software includes software for determining conditions needed for providing a stimulation signal or recording conditions of an obtained detection signal. With the use of this type of software, the computer 30 is not only capable of structuring the means of providing a stimulation signal to the cells and the means of processing the detection signal from the cells, but also is capable of controlling the optical observation means (the SIT camera and the image filing device) or the cell culturing means.
- [0047] In the following, particularly useful specifications for the software for measurement will be explained. On a computer screen directed to parameter setting, it is possible to determine complicated stimulation conditions by drawing a stimulation waveform on the screen using a keyboard or a mouse. Furthermore, recording conditions are determined such that 64 input channels, a sampling rate of 10 kHz, and continuous recording over several hours are enabled. In addition, the electrode which provides a stimulation signal or the electrode which draws out a detection signal from the cells can be specified by pointing to a microscope image displayed on the screen with a mouse or a pen. Besides, various conditions such as temperature or pH of the cell culturing means 40 can be determined by using the keyboard.
- [0048] A recording screen displays a spontaneous action potential or an evoked potential detected from the cells in real-time at a maximum of 64 channels. Furthermore, the recorded spontaneous action potential or the evoked potential can be displayed on top of a microscope image of cells. When the evoked potential is measured, the whole recording waveform is displayed. When the spontaneous action potential is measured, the recording waveform is displayed only when an oc-

currence of spontaneous action is detected by a spike detection function using a window discriminator or a waveform discriminator. When the recording waveform is displayed, measurement parameters (e.g., stimulation conditions, recording conditions, temperature, pH) at the time of recording are simultaneously displayed in real-time. There is also an alarm function provided in case when a temperature or pH goes beyond permissive limits.

[0049] On a computer screen for data analysis, FFT analysis, coherence analysis, and correlation analysis can be conducted. In addition, this screen has other functions, such as a single spike separation function using a waveform discriminator, a temporal profile display function, a topography display function, an electric current source density analysis function. Results of these analyses can be displayed on top of the microscope image stored in the image filing device.

[0050] When a stimulation signal is output from the above-configured computer 30, this stimulation signal is forwarded by way of the D/A converter 32 and an isolator 33 (for example, "BSI-2" manufactured by BAK ELECTRONICS CO., LTD.) to the cells. In other words, the stimulation signal is applied between two points selected from the 64 microelectrodes 11 in the integrated cell holding instrument 1. Then, an evoked potential arising between each of the microelectrodes 11 and a GND level (potential of culture solution) is input to the computer 30 via 64 channels of a sensitive amplifier 34 (for example, "AB-610J" manufactured by NIHON KODEN CO., LTD.) and the A/D converter 31. The amplification factor of the amplifier 34 is 100 dB, and the frequency band is from 0 to 10 kHz. However, when an evoked potential by a stimulation signal is measured, the frequency band is determined to be from 100 Hz to 10 kHz using a low cut filter.

[0051] Next, the cell culturing means 40 is provided with a temperature adjuster 41, a circulation means 42 of culture solution and a means 43 for supplying a mixed gas of air and carbon dioxide. Actually, the cell culturing means 40 can be comprised of a product equivalent to a microincubator such as "PDMI-2" and a product equivalent to a temperature controller such as "TC-202" (both products manufactured by MEDICAL SYSTEMS CO., LTD.), and a CO₂ bomb, for example, is used. This microincubator can control the temperature in the range of 0 to 50°C by a Peltier element, and this microincubator is capable of handing a liquid delivery speed of below 3.0 ml/min and an air supply speed of below 1.0 l/min. Alternatively, a microincubator integrated with a temperature controller (for example, "IMT2-IBSV" manufactured by OLYMPUS OPTICAL CO., LTD.) may be used.

[0052] A preferable embodiment of the cell potential measurement apparatus of this invention was explained above. However, the cell potential measurement apparatus of this invention is not limited to this embodiment only and can be implemented for example, in various other forms described in the following.

[0053] Although a means for providing a stimulation signal to cells is comprised of a computer and a D/A converter in the above-mentioned embodiment, this means may be comprised of a general purpose or a special purpose pulse signal generator. Here, the stimulation signal is preferably determined as a bipolar constant voltage pulse comprising a pair of positive and negative pulses for eliminating artifacts, that is, for preventing DC components from flowing. In addition, it is preferable to convert it to a constant electric current pulse for preventing the electric current from flowing excessively. For example, the stimulation signal is preferably comprised of a positive pulse with a pulse width of 100 μ sec, an interval of 100 μ sec, and a negative pulse of 100 μ sec, and it is preferable that the peak electric current of the positive-negative pulse is in the range of 30 to 200 μ A.

[0054] Furthermore, the installation of the cell culturing means 40 in the measurement apparatus enables continuous measurement over a long period of time. Alternatively, it is also possible to configure the apparatus such that sample cells are placed in an integrated cell holding instrument and cultured inside an incubator which is provided separately from the measurement apparatus, and such that the integrated cell holding instrument is taken out only for a comparatively short-term measurement from the incubator to be installed in the measuring apparatus. In this case, the cell culturing means 40 is not necessarily provided in the measurement apparatus.

[0055] By using the above-mentioned cell potential measurement apparatus, nerve cells or organs were actually cultured on the integrated cell holding instrument and the potential change accompanied by activities of the nerve cells or nerve organs was measured. An example of this measurement will be explained hereafter. Cerebral cortex sections of rats were used as the nerve organs, which were cultured according to a method which will be described later on in an embodiment.

[0056] It will be first referred to results of comparing a voltage waveform measured by means of an integrated cell holding instrument of this invention and a voltage waveform measured by means of a conventional general purpose glass electrode (electrode used for measurement of extracellular potential). Nerve organs which were cultured for 14 days were used as the sample. A stimulation signal was applied between two adjacent electrodes of a planar electrode comprising the integrated cell holding instrument, and a waveform of evoked potential change over time which was induced at 8 electrodes close to the two electrodes was measured. For the purpose of comparison, glass electrodes were sequentially transferred to the vicinity of the above-mentioned eight electrodes by using a three-dimensional micromanipulator, and the same voltage waveform was measured.

[0057] As a result of comparing the voltage waveform measured by using a planar electrode (integrated cell holding instrument) and the waveform measured by us-

ing the glass electrode at eight parts, it was clear that both waveforms were very similar at all the parts. Representative examples of these waveforms are shown in FIG. 10 (A) and FIG. 10 (B). FIG. 10 (A) shows a waveform measured by a planar electrode, and FIG. 10 (B) shows a waveform measured by a glass electrode. When both waveforms are compared, it is clear that there is a slight difference in frequency characteristics. Compared with the measurement using a planar electrode, the measurement using a glass electrode shows a small damage sustained to the follow-up property upon a rapid potential change. This is considered to result from a capacitance difference between a glass electrode and a planar electrode.

[0058] Next, an experiment was conducted to examine the relationship between progressive days of nerve organs cultured on an integrated cell holding instrument and the potential distribution arising from cell activities. Prior to culturing the cells, the surface of a planar electrode was covered with collagen gel for the purpose of enhancing the adhesive property of each electrode in the planar electrode to the cells. In other words, collagen gel with a thickness of less than 50 μm was formed on the surface of each electrode coated with platinum black and also on the surface of an insulation coating in the vicinity thereof as mentioned above. Then, on top of the collagen gel, and also where a microelectrode was present, a section of cerebral cortex of rats (thickness of less than 500 μm) was placed and cultured. Measurement results of the spontaneous potential are shown in FIGS. 11 (A) to 11 (C), and measurement results of the evoked potential at the time when a stimulation signal is provided are shown in FIG. 12.

[0059] FIG. 11 (A) shows a microscopic image of the sample cells and the microelectrodes, and waveforms of the spontaneous potential measured at seven electrode parts indicated as 1 to 7 on this image are shown in FIG. 11 (B) and FIG. 11 (C). FIG. 11 (B) is a waveform measured on the sixth day after culture, and FIG. 11 (C) is a waveform measured on the tenth day after culture.

The scale of the microscopic image, time of the measurement waveforms, and the scale of the voltage are indicated in the figure. According to the measurement results, it is confirmed, for example, that on the sixth day after culture, the spontaneous activities of the cells measured at each electrode are weak, and synchronic properties of the electrodes to each other can be hardly observed, whereas on the tenth day after culture, a large number of nerve cells become active simultaneously, indicating that the synchronic properties of the electrodes to each other increased.

[0060] FIG. 12 (A) also shows a microscopic view of the sample cells and the microelectrodes. Image processing, which is included in the software for measurement in the above-mentioned computer, was applied to draw an outline of the cells and positions of each electrode from the microscopic image onto the screen. Furthermore, the voltage waveform measured at each elec-

trode was displayed thereon, as shown in FIG. 12 (B) and FIG. 12 (C). FIG. 12 (B) shows a distribution of the evoked potential on the fifth day after culture, and FIG. 12 (C) shows the same on the tenth day after culture. A

- 5 pair of electrodes indicated on the upper right side with a + and - sign are electrodes applied with a stimulation signal. Right above a small square sign showing the position of each electrode, a waveform measured by this electrode is displayed. In these waveforms, a part where 10 a large vertical swing is observed on the left end is an artifact corresponding directly to the stimulation signal, and the potential change after the artifact indicates actual cell activities. As a result of these measurements, it is confirmed, for example, that on the fifth day after 15 culture, the cell activities are limited to a place which is comparatively close to the electrode positions applied with the stimulation signal, but on the tenth day after culture, the cell activities can be observed in a wide range and their scale (amplitude) becomes larger.
- 20 [0061] Next, examples of a suitable culture method for cerebral cortex slices will be explained.

1) Culture medium

25 [0062] The following additives were added to a culture medium in which Dulbecco modified Eagle's medium and HamF-12 medium were mixed in a volume ratio of 1:1 (media manufactured by GIBCO CO., LTD. 430-2500EB).

- 30 * glucose, GIBCO CO., LTD. 820-5023IN, 2.85 mg/L (totalling to 6 mg/L together with glucose contained originally in the above-mentioned culture medium)
* putrescine, SIGMA CO., LTD. P5780, 100 μM
35 * progesterone, SIGMA CO., LTD. P8783, 20 nM
* hydrocortisone, SIGMA CO., LTD. H0888, 20 nM
* sodium selenite, WAKO CO., LTD. 198-0319, 20 nM
* insulin, SIGMA CO.; LTD. I6634, 5 mg/L
* transferrin, SIGMA CO., LTD. T147, 100 mg/L
40 * sodium bicarbonate, SIGMA CO., LTD. 2.438 g/L
* addition of a suitable amount of IN HCl or IN NaOH to adjust to pH 7.4

45 [0063] After the above-mentioned additives were added, filtration and sterilization were conducted, and the culture medium was preserved at 4°C and ready to be used. This culture medium is hereinafter simply called "culture medium".

50 2) Structure of a well on a planar electrode

[0064] For the convenience of culturing nerve cells or nerve organs on a planar electrode, a polystyrene cylinder having an inner diameter of 22 mm, an outer diameter of 26 mm, and a height of 8 mm was adhered in the following steps.

- (a) On the bottom face of a polystyrene cylinder (in-

ner diameter 22 mm, outer diameter 26 mm, height 8 mm), a sufficient amount of a liquid silicon adhesive (DOW CORNING CO., LTD. 891 or SHIN-ETSU CHEMICAL CO., LTD. KE-42RTV) was applied. (b) The center of a glass substrate in the planar electrode and the center of the polystyrene cylinder were carefully matched and then adhered in this state. (c) By leaving it in an environment in which dust hardly enters for 24 hours, the adhesive was solidified. (d) After dipping in 70 % ethanol for 5 minutes, sterilization was conducted by air-drying inside a clean bench, after which the structure is then ready for processing the electrode surface.

3) Processing of the electrode surface

[0065] In order to enhance cell adhesive property on the surface of a planar electrode, collagen gel was formed on the surface of the electrode by the following method. All of these operations were conducted in a sterilized atmosphere.

(a) Solutions A, B, and C were prepared and iced.

A. 0.3 vol.% diluted hydrochloric acid collagen solution (pH 3.0, NITTA GELATIN CO., LTD. Cellmatrix Type I-A)

B. Solution comprising a mixture medium of Dulbecco modified Eagle's medium and HamF-12 medium mixed in a volume ratio of 1:1 (GIBCO CO., LTD. 430-2500EB), which is not provided with sodium bicarbonate and is made with a concentration 10 times higher than for an ordinary use, and then filtration and sterilization were conducted thereto.

C. 2.2 g of sodium bicarbonate and 4.77 g of HEPES (manufactured by GIBCO CO., LTD. 845-1344 IM) were dissolved in 100 mL of 0.05 N sodium hydroxide solution, and filtration and sterilization were conducted thereto.

(b) While cooling, the solutions A, B, and C were mixed at a volume ratio of 8:1:1. At this time, A and B were first mixed thoroughly and C was added afterwards to be mixed.

(c) In a well of a planar electrode which was cooled in advance to about 4°C, 1 mL of the mixed solution of (b) was injected little by little. After the entire electrode surface was covered, the mixed solution was removed as much as possible with a glass-Pasteur pipette. Through this operation, a coating of the mixed solution was formed on the electrode surface with a thickness of less than 50μm.

(d) By heating the planar electrode covered with the mixed solution coating at 37°C for 30 minutes, gelatinization of the mixed solution took place, and a

collagen gel matrix was formed.

(e) 1 mL of sterilized water was added into the well of the planar electrode, and about 5 minutes thereafter, the water was removed, thereby washing.

(f) The operation of Step (e) was repeated two more times (a total of 3 times).

(g) 1 mL of the culture medium (excluding insulin and transferrin) was injected little by little into the well of the planar electrode, and preserved inside a CO₂ incubator under the conditions of temperature 37°C, relative humidity 97% and higher, CO₂ concentration 5%, and air concentration 95%, which was then ready for use.

15 4) Culture of nerve cells or nerve organs

[0066] Generally speaking, culture forms can be divided into two types. That is, a dissociated cell culture of nerve cells and an organotypic slice culture of a nerve organ. Each form will be explained in the following.

4-1) Dissociated culture of cerebral visual cortex nerve cells of rats

[0067] The following operations were all performed in a sterilized atmosphere.

(a) Brains of fetuses of SD rats at 16-18 days of pregnancy were removed and immersed in iced Hanks' Balanced Salt Solution (manufactured by GIBCO CO., LTD. 450-1250EB).

(b) From the brains in the iced Hanks' Balanced Salt Solution, visual cortices were cut out and transferred to minimum essential medium liquid (manufactured by GIBCO CO., LTD. 410-1100EB).

(c) In the minimum essential medium liquid, the visual cortices were cut into as small pieces as possible, 0.2 mm square at maximum.

(d) The visual cortices cut into small pieces were placed in test tubes for centrifugal separation, and after washing with Hanks' Balanced Salt Solution free from calcium and magnesium three times, they were dispersed in a suitable volume of the same liquid.

(e) In the test tubes for centrifugal separation of Step (d), Hanks' Balanced Salt Solution free from calcium and magnesium with trypsin dissolved at 0.25 % was added to double the total volume. With gentle stirring, enzymatic processes were allowed to take place while the solution was constantly kept at 37 °C for 15 minutes.

(f)-To the culture medium shown in-1) (containing additives, hereinafter abbreviated as a culture medium), 10 vol.% of fetal calf serum was added, which is then placed in the test tubes for centrifugal separation subjected to Step (e) to further double the total volume. With a glass Pasteur pipette having a reduced diameter produced by fire-polishing

the tip end with a burner, gently repeating pipetting (about 20 times at maximum), the cells were unravelled.

(g) Centrifugation was carried out for 5 minutes at 9806.65 m/sec² (that is, 1000 g). Upon completion of centrifugation, the supernatant was discarded and the precipitate was suspended in the culture medium containing 5 vol.% of fetal cow serum.

(h) Step (g) was repeated two more times (a total of 3 times).

(i) The precipitate finally obtained was suspended in the culture medium containing 5 vol.% fetal cow serum, and using an erythrocytometer, the cell concentration in the suspension liquid was measured. After the measurement, using a similar culture medium, the cell concentration was adjusted to be 2 to 4 x 10⁶ cells/ml.

(j) A planar electrode which was preserved in a CO₂ incubator after subjected to the process of above steps 1-3) was taken out, the culture medium (free from insulin and transferrin) inside the well was removed, and 500μL of a culture medium containing 5 % of fetal cow serum was newly injected little by little. Furthermore, 100μL of the cell suspension liquid with the cell concentration adjusted according to Step (i) was gently added and again let stand in the CO₂ incubator.

(k) Three days after the performance of Step (j), one half the culture medium was replaced with a new one. For the replaced medium, the culture medium not containing fetal calf serum was used. By reducing the concentration of fetal calf serum, growth of cells other than nerve cells (for example, glial cells) can be suppressed.

(l) Thereafter, half of the medium was replaced in a similar manner every 1 to 2 days.

4-2) Culture method of a cerebral cortex section of rats

[0068]

(a) Brains of SD rats 2 days old were removed and immersed in iced Hanks' Balanced Salt Solution containing 0.25 vol.% of D-glucose.

(b) In the iced Hanks' Balanced Salt Solution containing 0.25 vol.% of D-glucose, cerebral meninges attached on the brain were removed using a sharp-edged pincer very carefully not to damage the cerebral cortex.

(c) About 500μm away from a callous body, a hemisphere of the cerebral cortex without the cerebral meninges was cut from the occipital lobe side to the frontal lobe side along the callous body by means of microscissors used for surgical operations of eyes.

(d) Subsequently, using the microscissors used for surgical operations of eyes, a cerebral cortex was cut out vertically to the cross-section of Step (c) with

a thickness of 200 to 300μm to create a section.

(e) The microscissors used for surgical operations of eyes was used further to adjust a size of the section to be about 1 x 1 mm.

(f) The planar electrode prepared in the above-mentioned "3) Processing of an electrode surface" was taken out from the CO₂ incubator, and the cerebral cortex section whose size was adjusted was sucked up with a pipette having a diameter of 2 mm and larger very gently not to damage the section, and then transferred into a culture well of the planar electrode.

(g) With a Pasteur pipette with the tip end fire-polished with a burner, the material was arranged on the electrode such that the layer structure of the cortex faces upward and is placed on the electrode, while being careful not to damage the cerebral cortex section.

(h) After the cerebral cortex section was placed on the planar electrode, the amount of the culture medium was adjusted so that a base of the section touched the culture medium and the top face was exposed to outside air.

(i) After adjusting the culture medium amount, the planar electrode was placed in a sterilized Petri dish, and about 5 ml of sterilized water at 37°C was injected little by little into the Petri dish to prevent the culture medium from drying, and again let stand in the CO₂ incubator.

(j) Thereafter, the medium was replaced with a new one once every day while attending to the amount of culture medium. The culture medium amount was determined to be the same as in Step (h).

Claims

1. A cell potential measurement apparatus for measurement of electric physiological characteristics of cells, comprising:

(A) an integrated cell holding instrument (1) provided with a plurality of microelectrodes (11) on a substrate plate (2), a cell holding part (6) for placing cells thereon, and an electric connection means (3, 4, 5) for providing an electric signal to said microelectrodes and for leading out an electric signal from said microelectrodes;

(B) a stimulation signal supply means (30, 32, 33)-to be connected to the electric connection means of said integrated cell holding instrument (1) for providing electric stimulation to said cells; and

(C) a signal processing means (30, 31, 34) to be connected to the electric connection means

- of said integrated cell holding instrument for processing an output signal arising from electric physiological activities of said cells, wherein said electric connection means includes a half-split holder (3, 4) which has a contact (9) touching an electric contact point (7) due to elastic deformation and fixes said substrate plate (2) by holding the plate at the top and bottom of the plate.
2. The cell potential measurement apparatus as in claim 1, further comprising an optical observation means (20) for observing the cells optically.
3. The apparatus as in claim 1 or 2, further comprising a cell culturing means (40) for maintaining an environment for culturing cells which are placed on said integrated cell holding instrument (1).
4. The cell potential measurement apparatus as in claim 3, wherein the cell culturing means (40) comprises a temperature adjustment means (41) for maintaining a constant temperature, a means (42) for circulating a culture solution, and a means (43) for supplying a mixed gas of air and carbon dioxide.
5. The apparatus as in any one of claims 1 to 4, wherein said integrated cell holding instrument (1) comprises a plurality of microelectrodes (11) arranged in a matrix form on the surface of a glass plate (13), conductive patterns (12) for drawing the microelectrodes, electric contact points (7) which are connected to edge parts of these conductive patterns (12), and an insulation coating (14) covering the surface of said conductive patterns (12), said cell holding part (6) being disposed in an area including said plurality of microelectrodes (11).
6. The apparatus as in any one of claims 1 to 5, wherein in said plurality of microelectrodes (11) comprise 64 electrodes arranged in 8 columns and 8 rows.
7. The apparatus as in any one of claims 1 to 6, wherein in said microelectrodes (11) each have an electrode area of $4 \times 10^2 \mu\text{m}^2$ to $4 \times 10^4 \mu\text{m}^2$.
8. The cell potential measurement apparatus as in any one of claims 1 to 7, wherein said electric connection means fixes said half-split holder (3, 4), and further comprises a printed circuit board (5) having an outside connection pattern which is connected to the contact of said holder via a connector.
9. The apparatus as in any one of claims 1 to 8, wherein the contact resistance of said electric contact point (7) with said contact (9) and the contact resistance of said contact (9) with said connector (5) are both less than 30 m ohm.
10. The apparatus as in any one of claims 1 to 9, wherein in said optical observation means (20) comprises an optical microscope (22), and an image pick-up device and an image display device (23) connected to the optical microscope.
11. The cell potential measurement apparatus as in claim 10, wherein said optical observation means further comprises an image storage device (24).
12. The apparatus as in any one of claims 1 to 11, wherein said stimulation signal supply means (30) comprises a pulse signal generator.
13. The apparatus as in any one of claims 1 to 12, wherein said signal processing means (30) comprises a multi-channel amplifier (34) which amplifies a detection signal arising from cell activities and a multi-channel display device which displays an amplified signal waveform in real-time.
14. The apparatus as in any one of claims 1 to 13, further comprising a computer (30) which outputs said stimulation signal via a D/A converter (32), and receives and processes an output signal arising from electric physiological activities of said cells via an A/D converter (31), said computer (30) controlling said optical observation means (20) and said cell culturing means (40).

Patentansprüche

1. Zellpotentialmeßvorrichtung zur Messung elektrophysiologischer Kennwerte von Zellen mit:
- (A) einem integrierten Zellhalteinstrument (1), versehen mit mehreren Mikroelektroden (11) auf einer Substratplatte (2), einem Zellhalteteil (6) zum Plazieren von Zellen darauf und einer elektrischen Verbindungseinrichtung (3, 4, 5) zum Zuführen eines elektrischen Signals zu den Mikroelektroden und zum Herausführen eines elektrischen Signals von den Mikroelektroden;
 - (B) einer Stimulationssignal-Zufuhreinrichtung (30, 32, 33), die mit der elektrischen Verbindungseinrichtung des integrierten Zellhalteinstruments (1) zu verbinden ist, zum elektrischen Stimulieren der Zellen; und
 - (C) einer Signalverarbeitungseinrichtung (30, 31-34), die mit der elektrischen Verbindungeinrichtung des integrierten Zellhalteinstruments zu verbinden ist, zum Verarbeiten eines Ausgangssignals als Ergebnis elektrophysiologischer Aktivitäten der Zellen, wobei die elektrische Verbindungseinrichtung einen geteilten Halter (3, 4) aufweist, der einen Kontakt (9) hat,

- der einen elektrischen Kontaktpunkt (7) infolge von elastischer Verformung berührt, und die Substratplatte (2) durch Halten der Platte an der Plattenoberseite und -unterseite fixiert.
2. Zellpotentialmeßvorrichtung nach Anspruch 1, ferner mit einer optischen Beobachtungseinrichtung (20) zum optischen Beobachten der Zellen.
3. Vorrichtung nach Anspruch 1 oder 2, ferner mit einer Zellkultivierungseinrichtung (40) zum Aufrechterhalten einer Umgebung zum Kultivieren von Zellen, die auf dem integrierten Zellhalteinstrument (1) plaziert sind.
4. Zellpotentialmeßvorrichtung nach Anspruch 3, wobei die Zellkultivierungseinrichtung (40) aufweist: eine Temperatureinstelleinrichtung (41) zum Aufrechterhalten einer konstanten Temperatur, eine Einrichtung (42) zum Zirkulierenlassen einer Kulturlösung und eine Einrichtung (43) zum Zuführen eines Mischgases aus Luft und Kohlendioxid.
5. Vorrichtung nach einem der Ansprüche 1 bis 4, wobei das integrierte Zellhalteinstrument (1) aufweist: mehrere Mikroelektroden (11), die in Matrixform auf der Oberfläche einer Glasplatte (13) angeordnet sind, Leitmuster (12) zum Herausführen der Mikroelektroden, elektrische Kontaktpunkte (7), die mit Kantenteilen dieser Leitmuster (12) verbunden sind, und eine Isolierbeschichtung (14), die die Oberfläche der Leitmuster (12) abdeckt, wobei das Zellhalteteil (6) in einem Bereich angeordnet ist, der die mehreren Mikroelektroden (11) aufweist.
6. Vorrichtung nach einem der Ansprüche 1 bis 5, wobei die mehreren Mikroelektroden (11) 64 Mikroelektroden aufweisen, die in 8 Spalten und 8 Reihen angeordnet sind.
7. Vorrichtung nach einem der Ansprüche 1 bis 6, wobei die Mikroelektroden (11) jeweils eine Elektrodenfläche von $4 \times 10^2 \mu\text{m}^2$ bis $4 \times 10^4 \mu\text{m}^2$ haben.
8. Zellpotentialmeßvorrichtung nach einem der Ansprüche 1 bis 7, wobei die elektrische Verbindungeinrichtung den geteilten Halter (3, 4) fixiert und ferner eine Leiterplatte (5) mit einem Außenverbindungsmuster aufweist, das mit dem Kontakt des Halters über einen Verbinder verbunden ist.
9. Vorrichtung nach einem der Ansprüche 1 bis 8, wobei der Kontaktwiderstand des elektrischen Kontaktpunkts (7) mit dem Kontakt (9) und der Kontaktwiderstand des Kontakts (9) mit dem Verbinder (5) beide unter $30 \text{ m}\Omega$ liegen.
10. Vorrichtung nach einem der Ansprüche 1 bis 9, wo-
- bei die optische Beobachtungseinrichtung (20) aufweist: ein optisches Mikroskop (22) sowie ein Bildaufnahmegerät und ein Bildanzeigegerät (23), die mit dem optischen Mikroskop verbunden sind.
11. Zellpotentialmeßvorrichtung nach Anspruch 10, wobei die optische Beobachtungseinrichtung ferner ein Bildspeichergerät (24) aufweist.
12. Vorrichtung nach einem der Ansprüche 1 bis 11, wobei die Stimulationssignal-Zufuhreinrichtung (30) einen Impulssignalgenerator aufweist.
13. Vorrichtung nach einem der Ansprüche 1 bis 12, wobei die Signalverarbeitungseinrichtung (30) aufweist: einen Mehrkanalverstärker (34), der ein Detektionssignal als Ergebnis von Zellaktivitäten verstärkt, und ein Mehrkanal-Anzeigegerät, das eine verstärkte Signalwellenform in Echtzeit anzeigt.
14. Vorrichtung nach einem der Ansprüche 1 bis 13, ferner mit einem Rechner (30), der das Stimulationssignal über einen D/A-Wandler (32) ausgibt sowie ein Ausgangssignal als Ergebnis elektrophysiologischer Aktivitäten der Zellen über einen A/D-Wandler (31) empfängt und verarbeitet, wobei der Rechner (30) die optische Beobachtungseinrichtung (20) und die Zellkultivierungseinrichtung (40) steuert.
- 30
- Revendications**
1. Appareil de mesure du potentiel cellulaire destiné à la mesure de caractéristiques physiologiques électriques de cellules, comprenant :
- (A) un instrument intégré de maintien de cellules (1) muni d'une pluralité de microélectrodes (11) sur une plaque de substrat (2), d'une partie de maintien de cellules (6) destinée à placer des cellules sur celle-ci, et d'un moyen de connexion électrique (3, 4, 5) destiné à fournir un signal électrique auxdites microélectrodes et à conduire à l'extérieur un signal électrique provenant desdites microélectrodes,
- (B) un moyen d'application de signal de stimulation (30, 32, 33) destiné à être relié au moyen de connexion électrique dudit instrument intégré de maintien de cellules (1), destiné à fournir une stimulation électrique auxdites cellules, et
- (C) un moyen de traitement de signal (30, 31, 34)-devant être relié au moyen de connexion électrique dudit instrument intégré de maintien de cellules, destiné à traiter un signal de sortie provenant d'activités physiologiques électriques desdites cellules, dans lequel ledit moyen de connexion électrique comprend un support séparé en deux (3, 4) qui comprend un contact

- (9) touchant un point de contact électrique (7) en raison d'une déformation élastique et qui fixe ladite plaque de substrat (2) en maintenant la plaque au niveau du haut et du bas de la plaque.
- 5
2. Appareil de mesure de potentiel cellulaire selon la revendication 1, comprenant en outre un moyen d'observation optique (20) destiné à observer optiquement les cellules.
- 10
3. Appareil selon la revendication 1 ou 2, comprenant en outre un moyen de culture de cellules (40) destiné à maintenir un environnement en vue de cultiver les cellules qui sont placées sur ledit instrument intégré de maintien de cellules (1).
- 15
4. Appareil de mesure de potentiel cellulaire selon la revendication 3, dans lequel le moyen de culture de cellules (40) comprend un moyen d'ajustement de température (41) destiné à maintenir une température constante, un moyen (42) destiné à faire circuler une solution de culture et un moyen (43) destiné à fournir un gaz mixte d'air et de dioxyde de carbone.
- 20
5. Appareil selon l'une quelconque des revendications 1 à 4, dans lequel ledit instrument intégré de maintien de cellules (1) comprend une pluralité de microélectrodes (11) disposées suivant une forme de matrice sur la surface d'une plaque de verre (13), des motifs conducteurs (12) destinés à dessiner les microélectrodes, des points de contact électriques (7) qui sont reliés aux parties de bord de ces motifs conducteurs (12), et un revêtement d'isolation (14) recouvrant la surface desdits motifs conducteurs (12), ladite partie de maintien de cellules (6) étant disposée dans une zone comprenant ladite pluralité de microélectrodes (11).
- 25
6. Appareil selon l'une quelconque des revendications 1 à 5, dans lequel ladite pluralité de microélectrodes (11) comprend 64 électrodes disposées en 8 colonnes et 8 rangées.
- 30
7. Appareil selon l'une quelconque des revendications 1 à 6, dans lequel lesdites microélectrodes (11) présentent chacune une surface d'électrode de $4 \times 10^2 \mu\text{m}^2$ à $4 \times 10^4 \mu\text{m}^2$.
- 35
8. Appareil de mesure de potentiel cellulaire selon l'une quelconque des revendications 1 à 7, dans lequel ledit moyen de connexion électrique fixe ledit support séparé en deux (3, 4) et comprend en outre une carte à circuit imprimé (5) comportant un motif de connexion extérieur qui est connecté au contact dudit support par l'intermédiaire d'un connecteur.
- 40
9. Appareil selon l'une quelconque des revendications 1 à 8, dans lequel la résistance de contact dudit point de contact électrique (7) avec ledit contact (9) et la résistance de contact dudit contact (9) avec ledit connecteur (5) sont toutes les deux inférieures à 30 milliohms.
- 45
10. Appareil selon l'une quelconque des revendications 1 à 9, dans lequel ledit moyen d'observation optique (20) comprend un microscope optique (22), et un dispositif de saisie d'image ainsi qu'un dispositif d'affichage d'image (23) reliés au microscope optique.
- 50
11. Appareil de mesure de potentiel cellulaire selon la revendication 10, dans lequel ledit moyen d'observation optique comprend en outre un dispositif de mémorisation d'image (24).
- 55
12. Appareil selon l'une quelconque des revendications 1 à 11, dans lequel ledit moyen d'application de signal de stimulation (30) comprend un générateur de signal d'impulsion.
13. Appareil selon l'une quelconque des revendications 1 à 12, dans lequel ledit moyen de traitement de signal (30) comprend un amplificateur à plusieurs canaux (34) qui amplifie un signal de détection provenant d'activités des cellules et un dispositif d'affichage à plusieurs canaux qui affiche une forme d'onde de signal amplifiée en temps réel.
14. Appareil selon l'une quelconque des revendications 1 à 13, comprenant en outre un ordinateur (30) qui fournit en sortie ledit signal de stimulation par l'intermédiaire d'un convertisseur numérique vers analogique (N/A) (32), et reçoit et traite un signal de sortie provenant d'activités physiologiques électriques desdites cellules par l'intermédiaire d'un convertisseur analogique vers numérique (A/N) (31), ledit ordinateur (30) commandant ledit moyen d'observation optique (20) et ledit moyen de culture de cellules (40).

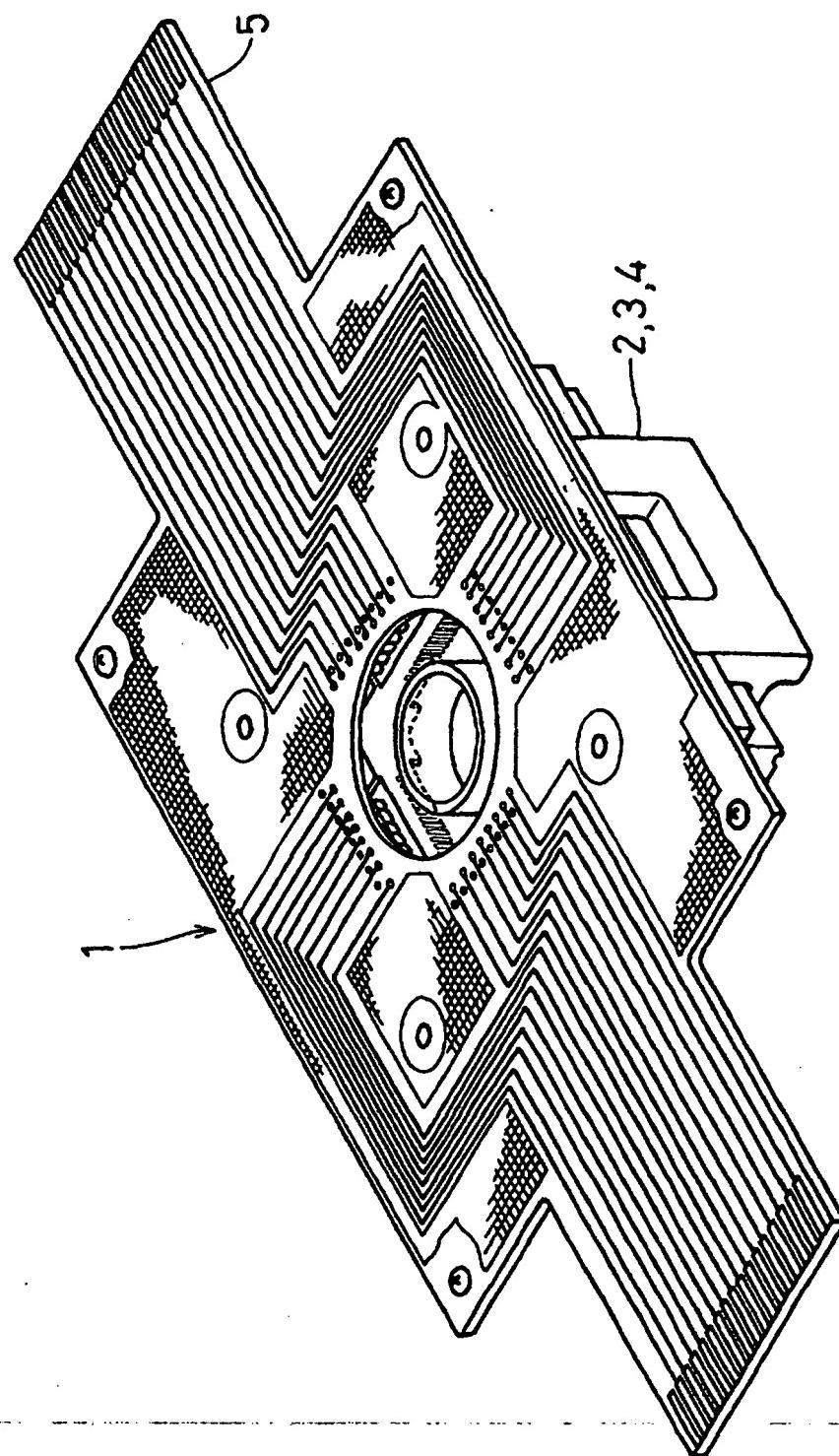
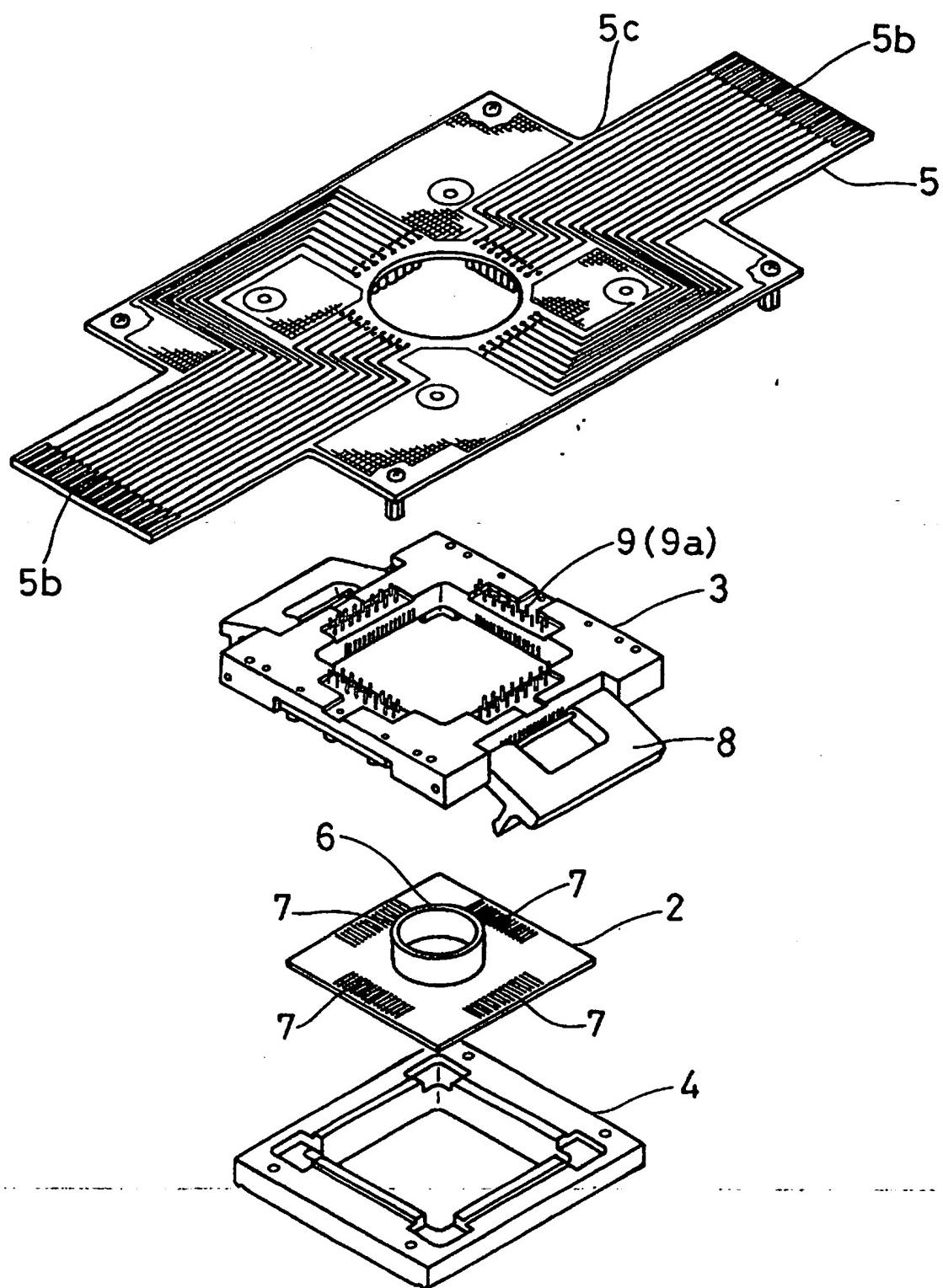


FIG. 1

FIG. 2



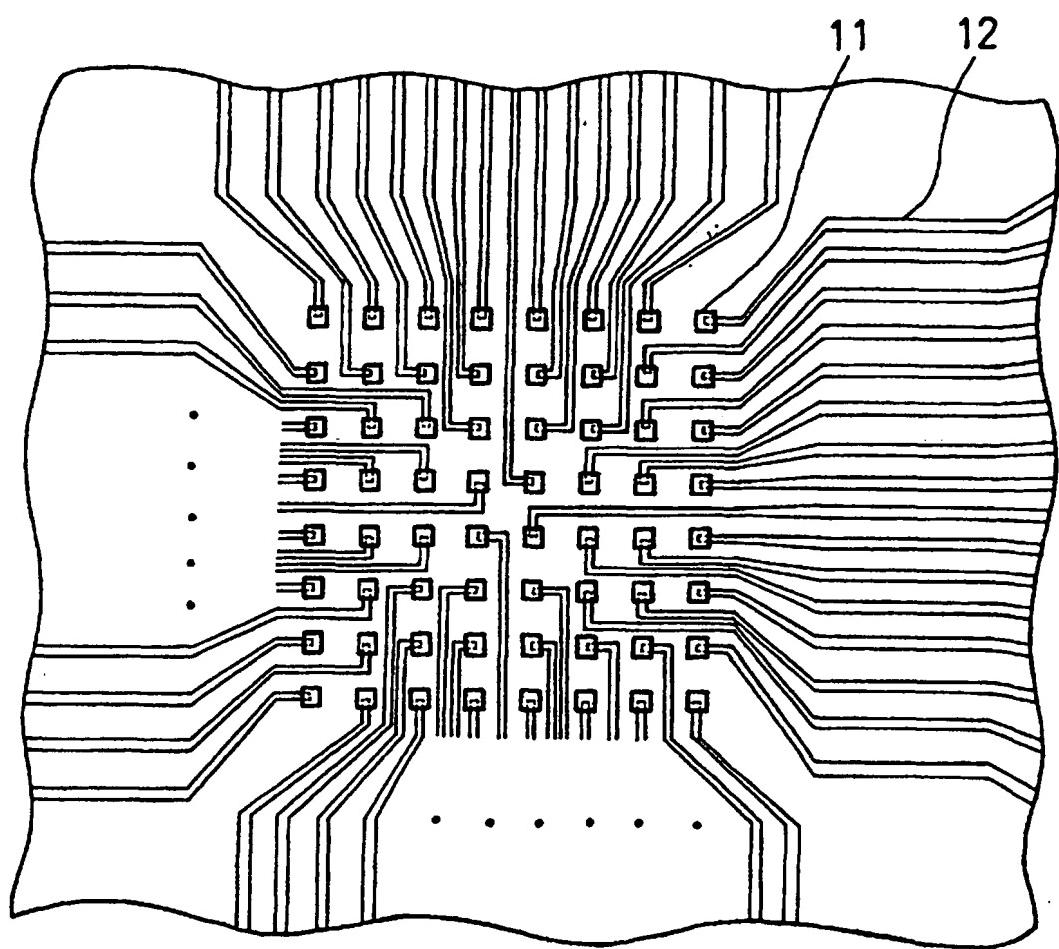


FIG. 3

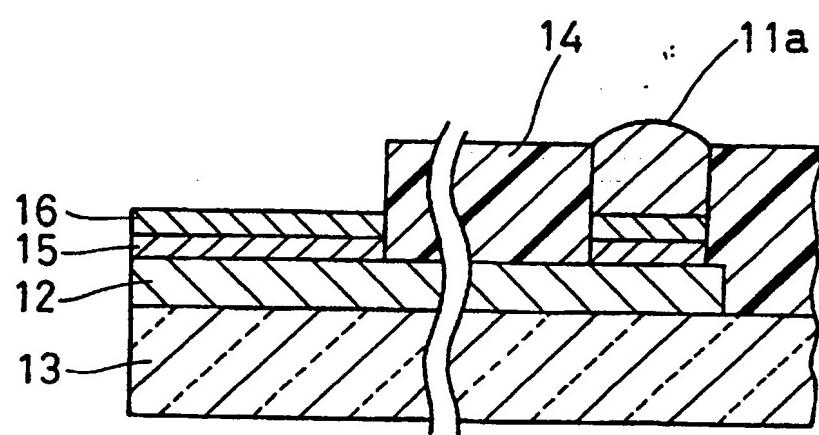


FIG. 4

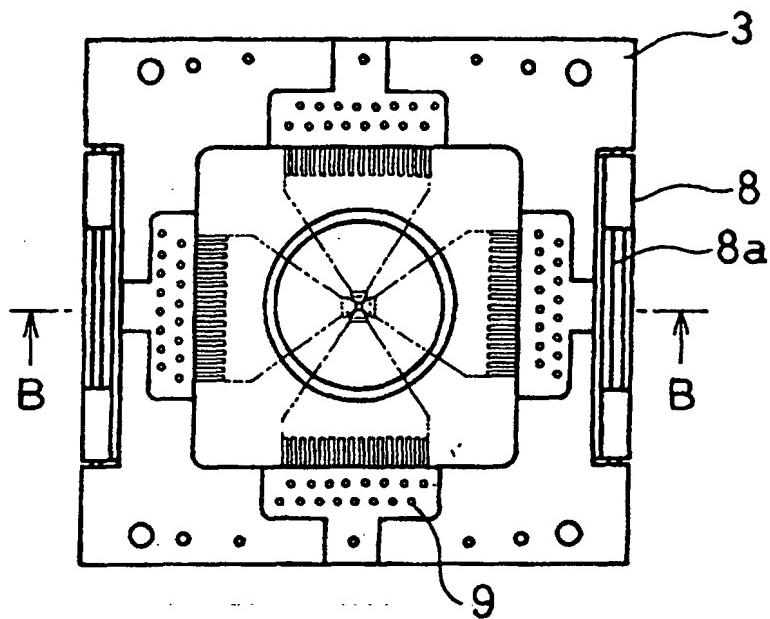


FIG. 5 (A)

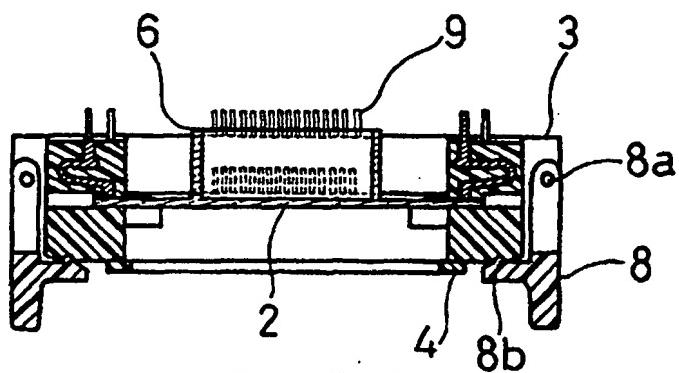


FIG. 5 (B)

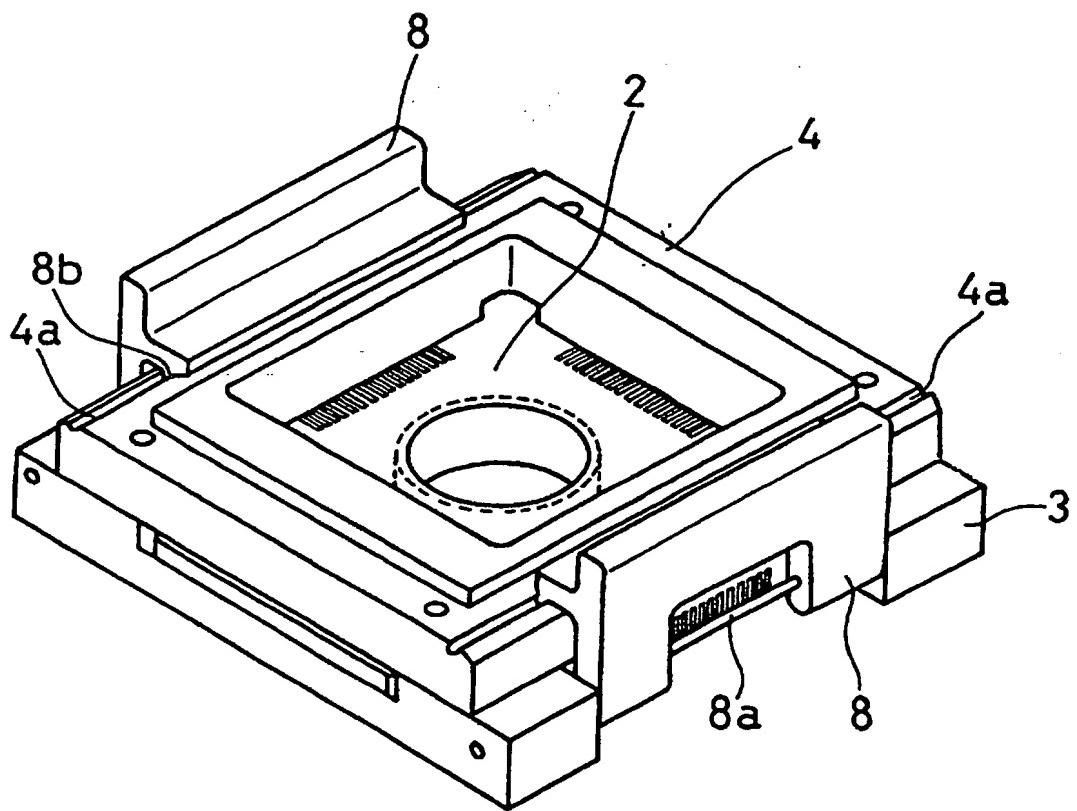


FIG. 6

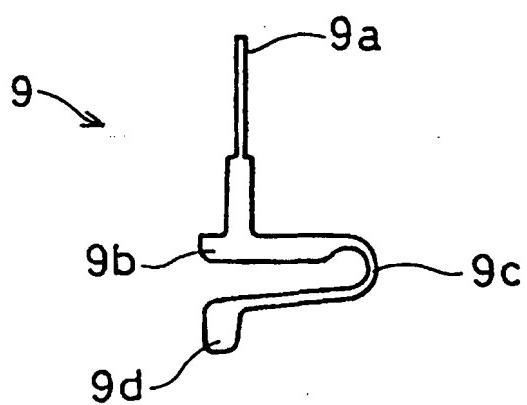
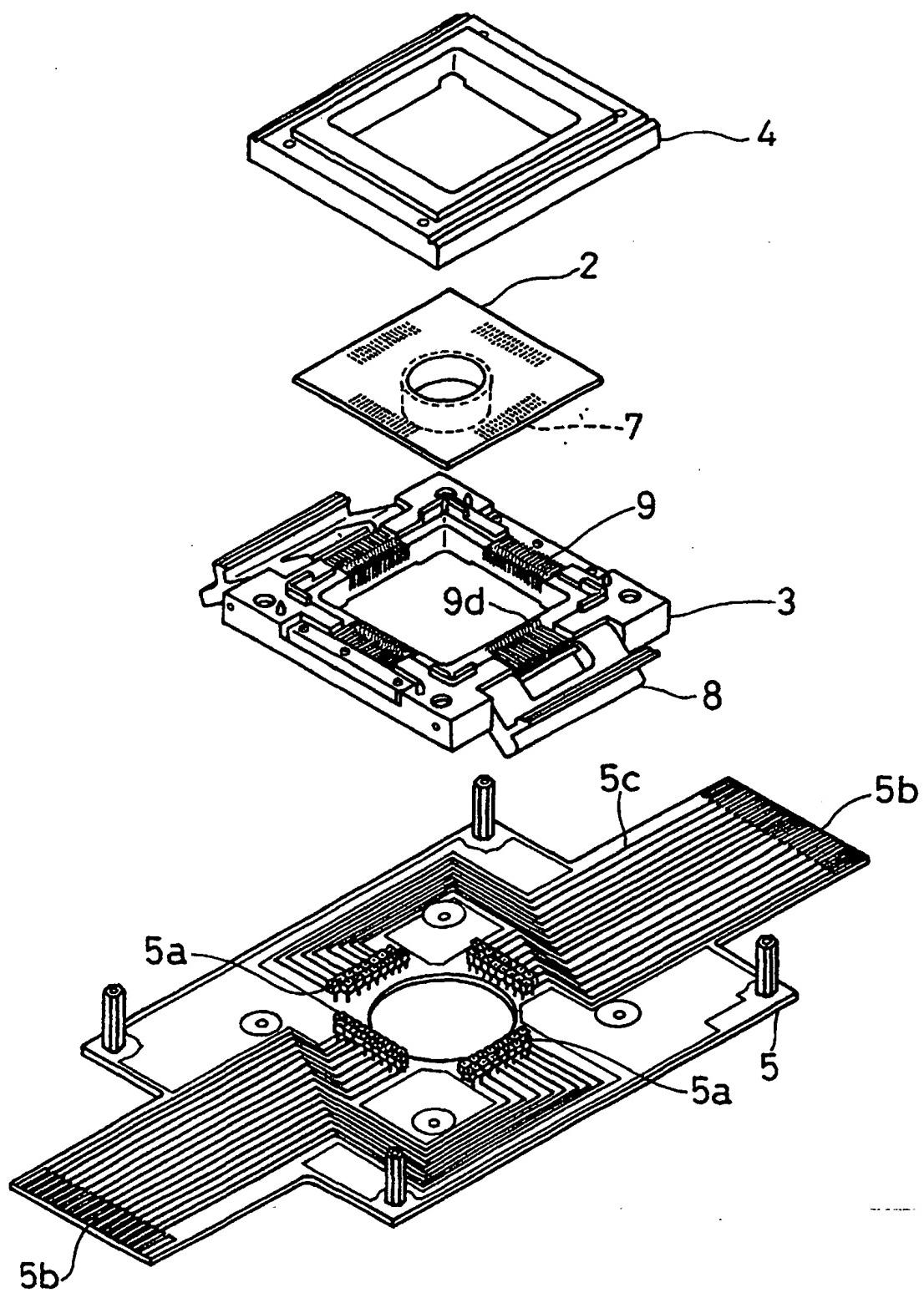


FIG. 7

FIG. 8



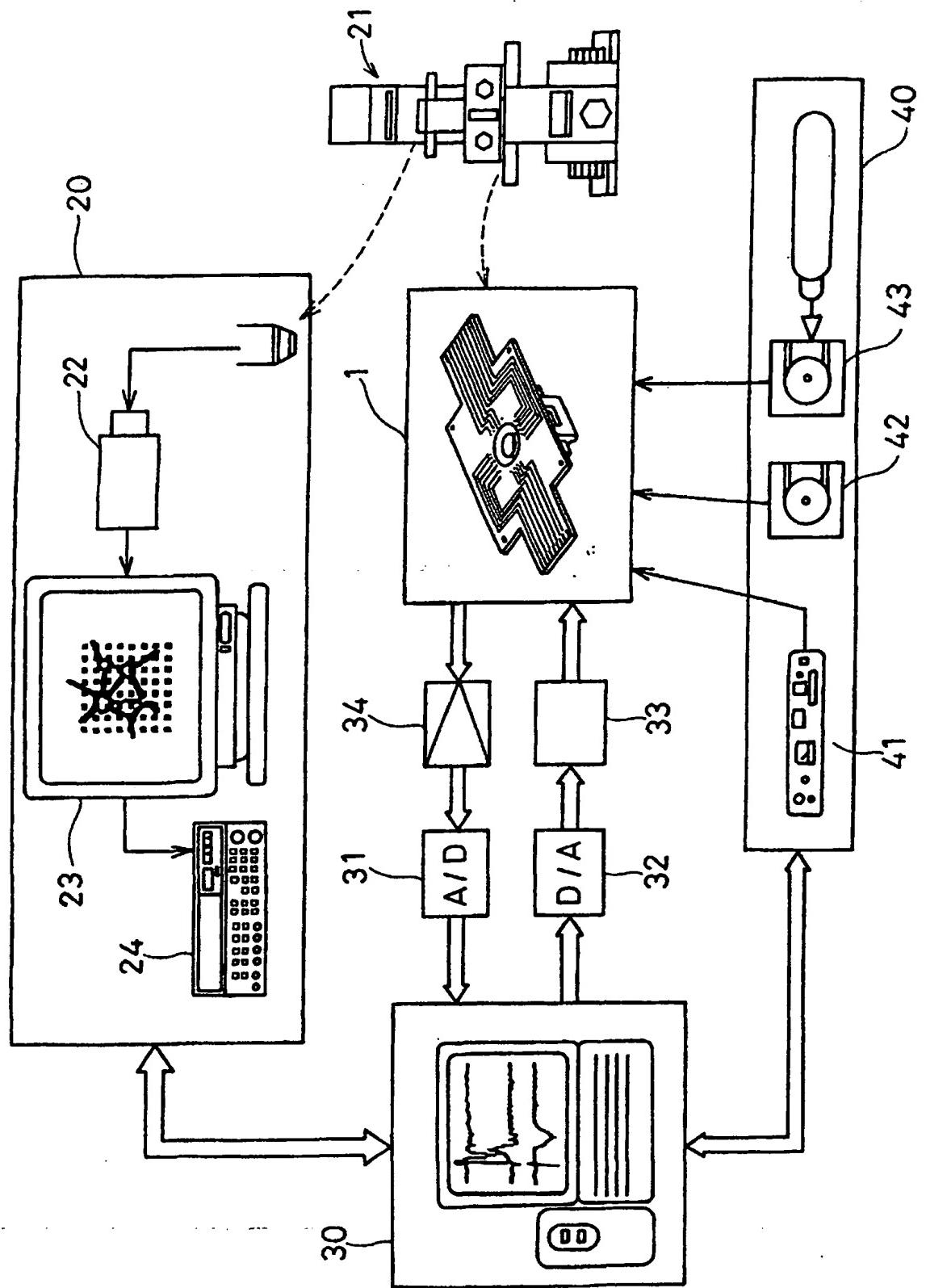


FIG. 9

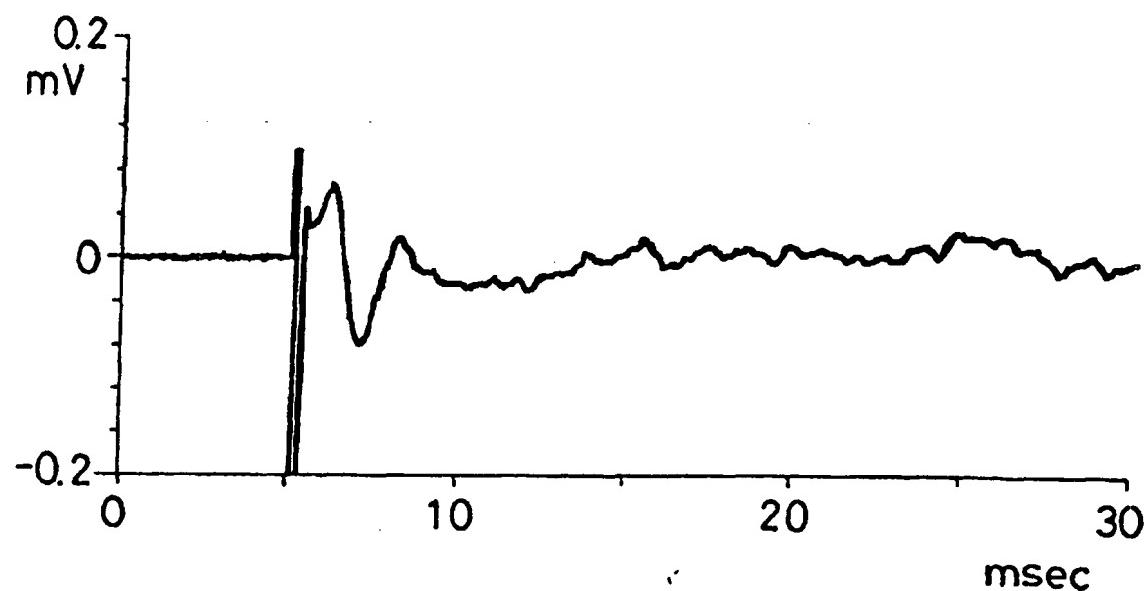


FIG.10 (A)

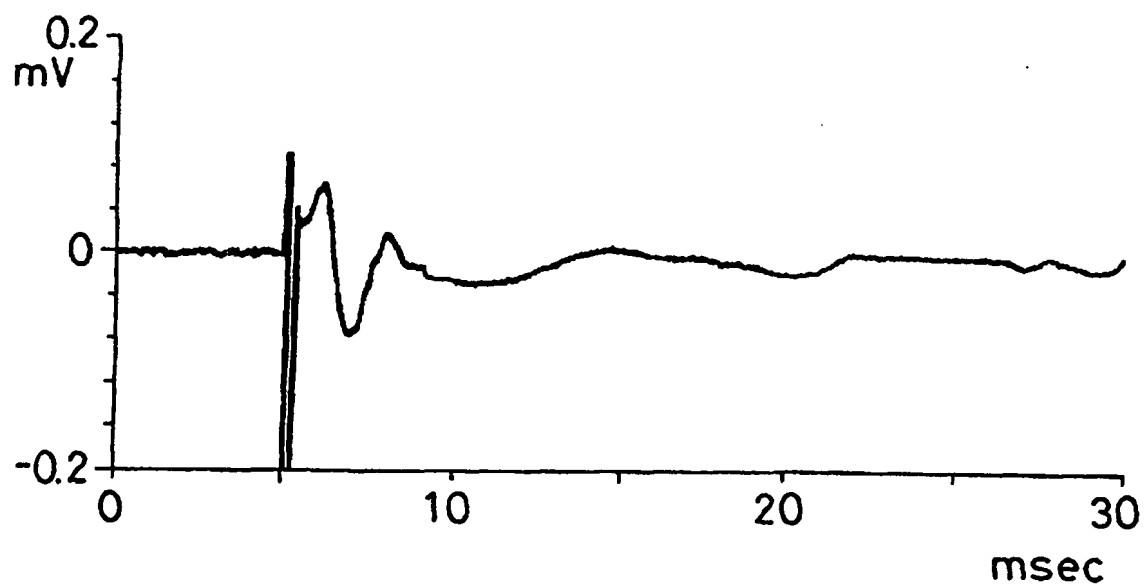


FIG.10(B)

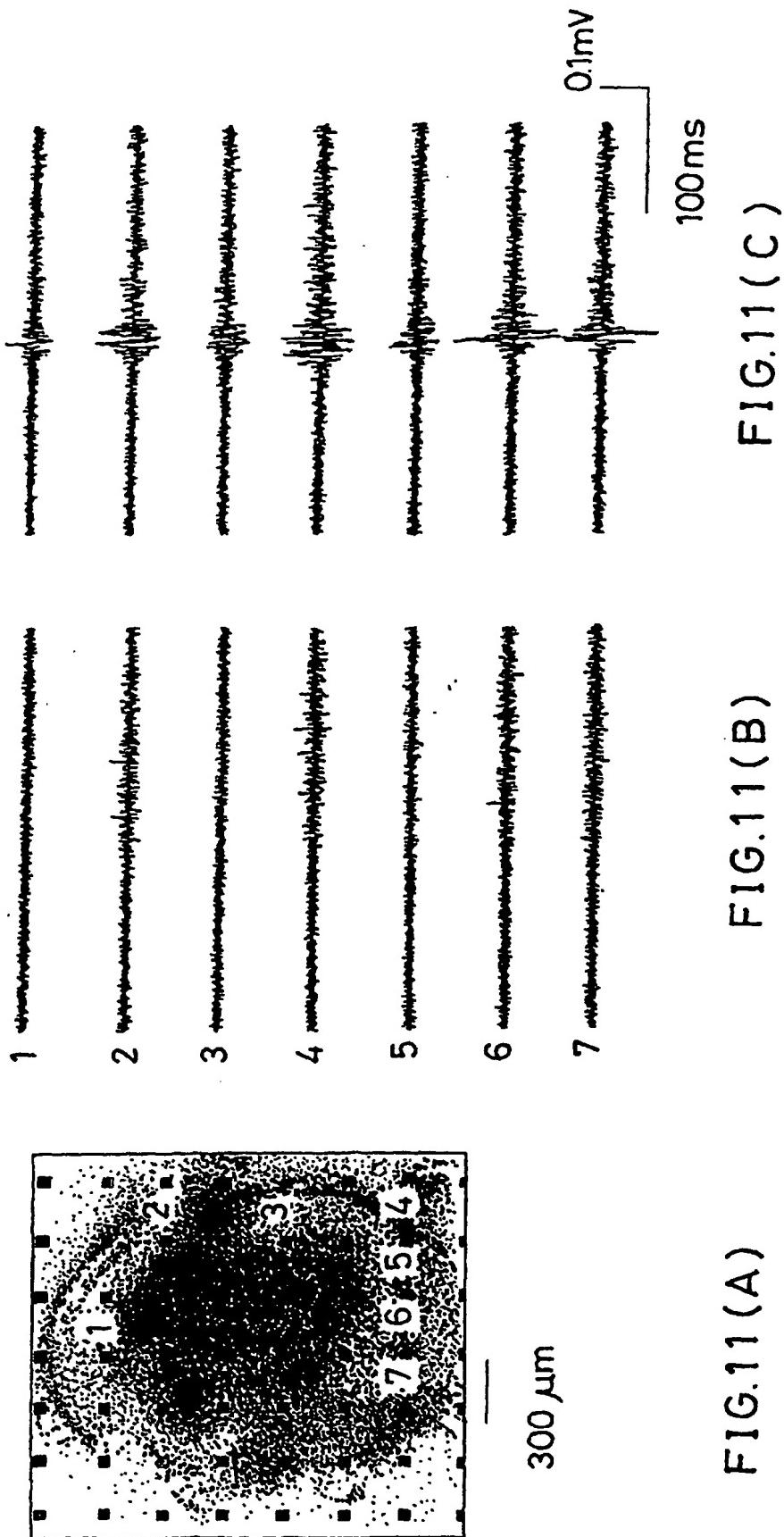


FIG.11 (A)

FIG.11 (B)

FIG.11 (C)

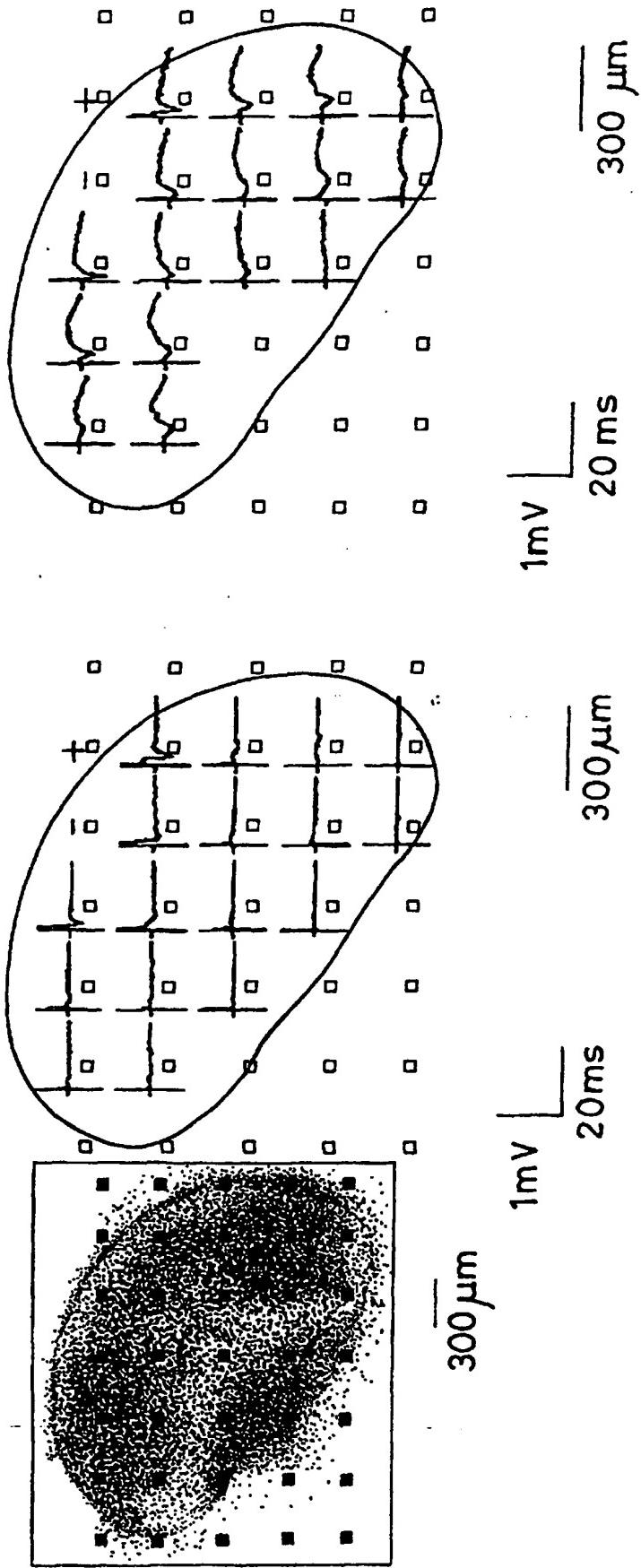


FIG.12 (A)

FIG.12 (B)

FIG.12 (C)

(19)



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(54) MESOSCALE SAMPLE PREPARATION DEVICE AND SYSTEMS FOR DETERMINATION AND PROCESSING OF ANALYTES

MINIATURISIERTE PROBENVORBEREITUNGSVORRICHTUNGEN SOWIE SYSTEME ZUR
FESTSTELLUNG UND BEHANDLUNG VON ANALYTEN

DISPOSITIF DE PREPARATION D'ECHANTILLONS MESOECHELLE ET SYSTEMES POUR LA
DETERMINATION ET LE TRAITEMENT DES ANALYSATS

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Description**BACKGROUND OF THE INVENTION**

- 5 [0001] This invention relates to sample preparation devices having small dimensions for facilitating the efficient preparation of microvolume test samples, e.g., of whole blood, for the determination and/or processing of analytes present therein. The present invention also relates to test systems including such devices, together with devices of similar dimensions which are designed, for example, to perform various assay protocols as well as analyses involving amplification of pre-selected polynucleotides, such as polymerase chain reaction (PCR).
- 10 [0002] In recent decades the art has developed a large number of protocols, test kits, and devices for conducting analyses on biological samples for various diagnostic and monitoring purposes. Immunoassays, immunometric assays, agglutination assays, analyses involving polynucleotide amplification reactions, various ligand-receptor interactions, and differential migration of species in a complex sample all have been used to determine the presence or quantity of various biological molecules or contaminants, or the presence of particular cell types.
- 15 [0003] Recently, small, disposable devices have been developed for handling biological samples and for conducting certain clinical tests. Shoji et al. reported the use of a miniature blood gas analyzer fabricated on a silicon wafer. Shoji et al., Sensors and Actuators, 15: 101-107 (1988). Sato et al. reported a cell fusion technique using micromechanical silicon devices. Sato et al., Sensors and Actuators, A21-A23: 948-953 (1990). Ciba Corning Diagnostics Corp. (USA) has manufactured a microprocessor-controlled laser photometer for detecting blood clotting.
- 20 [0004] Micromachining technology originated in the microelectronics industry. Angell et al., Scientific American, 248: 44-55 (1983). Micromachining technology has enabled the manufacture of microengineered devices having structural elements with minute dimensions, ranging from tens of microns (the dimensions of biological cells) to nanometers (the dimensions of some biological macromolecules). Most experiments reported to date involving such small structures have involved studies of micromechanics, i.e., mechanical motion and flow properties. The potential capability of such devices has not been exploited fully in the life sciences.
- 25 [0005] Brunette (Exper. Cell Res., 167: 203-217 (1986) and 164: 11-26 (1986)) studied the behavior of fibroblasts and epithelial cells in grooves in silicon, titanium-coated polymers and the like. McCartney et al. (Cancer Res., 41: 3046-3051 (1981)) examined the behavior of tumor cells in grooved plastic substrates. LaCelle (Blood Cells, 12: 179-189 (1986)) studied leukocyte and erythrocyte flow in microcapillaries to gain insight into micro-circulation. Hung and Weissman reported a study of fluid dynamics in micromachined channels, but did not produce data associated with an analytical device. Hung et al., Med. and Biol. Engineering, 9: 237-245 (1971); and Weissman et al., Am. Inst. Chem. Eng. J., 17: 25-30 (1971). Columbus et al. utilized a sandwich composed of two orthogonally orientated v-grooved embossed sheets in the control of capillary flow of biological fluids to discrete ion-selective electrodes in an experimental multi-channel test device. Columbus et al., Clin. Chem., 33: 1531-1537 (1987). Masuda et al. and Washizu et al. have reported the use of a fluid flow chamber for the manipulation of cells (e.g., cell fusion). Masuda et al., Proceedings IEEE/IAS Meeting, pp. 1549-1553 (1987); and Washizu et al., Proceedings IEEE/IAS Meeting, pp. 1735-1740 (1988). The art has not fully explored the potential of using microengineered devices for the determination of analytes in fluid samples, particularly in the area of biological analyses.
- 30 [0006] Biological analyses utilizing polynucleotide amplification techniques are well known (See e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, pp. 14.1-14.35). One such technique is PCR amplification, which can be performed on a DNA template using a thermostable DNA polymerase, e.g., Taq DNA polymerase (Chien et al., J. Bacteriol., 127: 1550 (1976)), nucleoside triphosphates, and two oligonucleotides with different sequences, complementary to sequences that lie on opposite strands of the template DNA and which flank the segment of DNA that is to be amplified ("primers"). The reaction components are cycled between a higher temperature (e.g., 94°C) for dehybridizing double stranded template DNA, followed by lower temperatures (e.g., 65°C) for annealing and polymerization. A repeated reaction cycle between dehybridization, annealing and polymerization temperatures provides approximately exponential amplification of the template DNA. Machines for performing automated PCR chain reactions using a thermal cycler are available (Perkin Elmer Corp.)
- 35 [0007] PCR amplification has been applied to the diagnosis of genetic disorders (Engelke et al., Proc. Natl. Acad. Sci., 85: 544 (1988), the detection of nucleic acid sequences of pathogenic organisms in clinical samples (Ou et al., Science, 239: 295 (1988)), the genetic identification of forensic samples, e.g., sperm (Li et al., Nature, 335: 414 (1988)), the analysis of mutations in activated-oncogenes (Farr et al., Proc. Natl. Acad. Sci., 85: 1629 (1988))-and-in many aspects of molecular cloning (Oste, BioTechniques, 6: 162 (1988)). PCR assays can be used in a wide range of applications such as the generation of specific sequences of cloned doublestranded DNA for use as probes, the generation of probes specific for uncloned genes by selective amplification of particular segments of cDNA, the generation of libraries of cDNA from small amounts of mRNA, the generation of large amounts of DNA for sequencing, and the analysis of mutations. There is a need for convenient, rapid systems for performing polynucleotide amplification, which may be used clinically in a wide range of potential applications in clinical tests such as tests for paternity, and for genetic

and infectious diseases.

[0008] Current analytical techniques utilized for the determination of microorganisms are rarely automated, usually require incubation in a suitable medium to increase the number of organisms, and generally employ visual and/or chemical methods to identify the strain or sub-species of interest. The inherent delay in such methods frequently necessitates medical intervention prior to definitive identification of the nature of an infection. In industrial, public health or clinical environments, such delays may have unfortunate consequences. There is a need for convenient systems for the rapid detection of microorganisms.

[0009] It is an object of the present invention to provide sample preparation devices for use with related analytical devices which enable rapid and efficient analysis of sample fluids, based on very small volumes, and determination of substances present therein at very low concentrations. Another object is to provide easily mass produced, disposable, small (e.g., less than 1 cc in volume) devices having microfabricated structural elements capable of facilitating rapid, automated analyses of preselected molecular or cellular analytes, including intracellular molecules, such as DNA, in a range of biological and other applications. It is a further object of the invention to provide a variety of such devices that individually can be used to implement a range of rapid clinical tests, e.g., tests for viral or bacterial infection, genetic screening, sperm motility, blood parameters, contaminants in food, water, or body fluids, and the like.

SUMMARY OF THE INVENTION

[0010] The present invention provides a microfabricated sample preparation device which conveniently provides microvolume fractions of test sample comprising particulate components, e.g., cells, for various biological and other analyses. The invention further provides analytical systems which include the microfabricated sample preparation device of the invention together with a microfabricated analyte detection device, e.g., an immunoassay device, and/or a microfabricated device for carrying out polynucleotide amplification.

[0011] The sample preparation device of the present invention is defined in claim 1 hereinafter.

[0012] According to one embodiment of the invention, the flow path has at least one mesoscale dimension and the separator comprises a region of restricted flow in the flow path, which is formed by at least one passageway having at least one mesoscale dimension that is smaller than the least mesoscale dimension of the flow path and sufficiently small to separate particulate components from the sample fluid.

[0013] The sample preparation device of the invention can be made using known microfabrication techniques, with the flow path and the flow channel being formed in a surface of a solid substrate. In a preferred embodiment, the surface of the substrate in which the structural elements are formed is enclosed by a cover, such as a transparent glass or plastic cover, adhered to such surface.

[0014] The mesoscale sample preparation device of the present invention is specially adapted for use in conjunction with the mesoscale detection devices which are the subject of co-pending U.S. Serial No. 07/877,702, available in the file of WO 93122053 which claims convention priority from it and was published on 11th November 1993 and/or the mesoscale polynucleotide amplification devices which are the subject of copending U.S. Serial No. 08/308,199. A brief indication of the contents of U.S. Serial No. 08/308199 is given later in the text of this application.

[0015] The mesoscale devices described above can be used in various combinations to function as an analytical system, as will be described in further detail below. In one embodiment, the devices may be utilized for analyses of a cell-containing test sample. The test sample fractions provided by the sample preparation device of the present invention may be analyzed serially or essentially simultaneously.

[0016] The mesoscale detection devices, which enable the determination of various analytes of interest, comprise a solid substrate microfabricated to define a sample inlet port and a mesoscale flow system which includes an analyte detection region in fluid communication with the inlet port and, optionally, a flow channel interconnecting the inlet port and the analyte detection region. At least one of the analyte detection region and the sample flow channel, when present, has at least one mesoscale dimension. The analyte detection region is provided with a reagent which interacts with the analyte of interest, resulting in a detectable product which is determinative of the analyte. In one embodiment, the reagent is a binding substance, optionally immobilized in the detection region, either on a stationary or mobile support, for specifically binding the analyte. Also included is a detector for detecting the aforementioned product, which allows determination of the analyte in the test sample.

[0017] The mesoscale polynucleotide amplification device comprises a solid substrate that is microfabricated to define a sample inlet port and a mesoscale flow system, which includes a polynucleotide amplification region in fluid communication with the inlet port of the device, and, optionally, a flow channel interconnecting the inlet port and the polynucleotide amplification region. At least one of the polynucleotide amplification region and the sample flow channel, when the latter is present, has at least one mesoscale dimension. Lysing means is also provided in a sample flow channel upstream of the polynucleotide amplification region for lysing cell components of a biological test sample. Such devices may be utilized to implement PCR, in which case the polynucleotide amplification region contains appropriate reagents and means is provided for thermally cycling the reagents, such that, in each cycle, the temperature

is controlled to dehybridize double stranded polynucleotides, anneal the primers to single stranded polynucleotide, and synthesize amplified polynucleotide between the primers.

[0018] The individual analytical devices described herein are within the scope of the present invention, when they are used in conjunction with the sample preparation device of the invention.

[0019] The devices described above will normally be used with an appliance that functions as a holder for the devices and which mates one or more ports on the devices with one or more flow lines in the appliance. A test sample, such as whole blood, containing an analyte of interest may be applied to the inlet of the sample preparation device after which an impellent, such as a pump, which may be incorporated in the appliance or in the device itself, is employed to cause the sample to flow along the flow path and through the separation zone. Test sample which is free of particulate components is transferred from the sample preparation device to the analyte detection device, the outlet of the former being in fluid communication with the inlet port of the latter. Particulate components, such as blood cells or other formed bodies, remaining in the separation zone can be discharged from the separation zone, and transferred to the polynucleotide amplification device via the discharge section of the flow channel of the sample preparation device, which is in fluid communication with the inlet port of the polynucleotide amplification device. Alternatively, the test sample may be injected into the sample preparation device, or the sample may enter the mesoscale sample preparation device through the inlet by capillary action. Optionally, depending on the analytical protocol being carried out in the devices described above, the appliance may also be designed to inject into the devices reagents, such as labelled binding substances, polynucleotide amplification reagents, buffers, or any other reagent required to carry out the desired analysis.

[0020] The device and systems of the invention may be used to implement a variety of automated, sensitive and rapid clinical tests including the analysis of cells or molecules or for monitoring reactions or cell growth. Essentially any test involving determination of the presence or concentration of a molecular or ionic analyte, the presence of a particular cell type or the presence of a gene or recombinant DNA sequence in a cell can be implemented to advantage using the device and analytical systems of the present invention. These mesoscale devices can provide a rapid chemical test for the detection of pathogenic bacteria or viruses. The devices can also provide a rapid test for the presence or concentration of blood constituents, such as hormones. Additional useful applications include, but are not limited to, a range of other biological assays, such as blood type testing.

[0021] The device and systems of the invention may be readily sterilized prior to use. Tests performed using the device and systems of the invention may be completed rapidly, and at the conclusion of the test the devices can be discarded, which beneficially prevents contamination between samples, entombs potentially hazardous material, produces only microvolumes of waste fluid for disposal and enables inexpensive analyses.

[0022] Additional advantages and features of the present invention are set forth in, and will be apparent to those skilled in the art from the detailed description of the invention presented below, considered in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023]

[0023] FIGURE 1 is a perspective view of a diagrammatic representation of a sample preparation device of the invention, as seen through a transparent cover.

FIGURES 2 and 3 show fragmentary plan views of different embodiments of a microfabricated restricted flow (filter-type) separator within the flow path through a portion of a sample preparation device, the separator having a series of passageways restricting flow of the test sample through the flow path.

FIGURE 4 is a schematic illustration, in cross-section, of a sample preparation device of the invention combined with an appliance which serves to hold the device and to regulate fluid flow through the device.

FIGURE 5 is a plan view of a diagrammatic representation of the same device shown in FIGURE 1, the respective outlets of which are in fluid communication with first and second microfabricated analytical structures which are designed to perform separate analyses on the sample fractions provided by the sample preparation device.

FIGURES 6A and 6B are schematic illustrations, in cross-section, of a sample preparation device of the invention with the outlet of the flow path from the separation zone in fluid communication with the sample inlet of an analytical device for implementing various assay protocols. Both devices are shown in combination with an appliance which serves to hold the devices, regulate fluid flow through the devices, and, in the embodiment shown in FIGURE 6A, detect pressure differentials at preselected locations along the course of fluid flow through the devices. FIGURE 6A shows the devices abutting end-to-end; and FIGURE 6B shows a stacked arrangement of the devices.

FIGURE 7 is a schematic illustration, in cross-section, of a sample preparation device of the invention with the outlet of the carrier fluid flow channel in fluid communication with the sample inlet of an analytical device for performing polynucleotide amplification. Both devices are shown in combination with an appliance which serves to

hold the devices, regulate fluid flow through the devices and detect pressure differentials at preselected locations along the course of fluid flow through the devices.

FIGURES 8A and 8B show, in plan view, diagrammatic illustrations of two analytical devices intended for use with the sample preparation device of the invention. The device of **FIGURE 8A** has two mesoscale flow systems, each one including inlet ports interconnected by a flow channel to a single chamber for analyte capture and, optionally, detection. **FIGURE 8B** shows a similar design for performing enzyme immunoassays and having dual capture chambers. An analyte of interest, such as a protein, may be captured in the first chamber, e.g., by a suitable immunocapture reagent, labelled with an antibody-enzyme conjugate and exposed to a chromogenic substrate. The enzyme converts the substrate to a chromophore which is captured, e.g., by a suitable immunocapture reagent, in the second chamber which concentrates the chromophore and reduces background signal. The second chamber may optionally be used for detection of the chromophore, as well.

FIGURE 9 is a plan view of a diagrammatic representation of a microfabricated analytical device intended for use with the sample preparation device of the invention. The analytical device includes a set of tortuous channels which enable the timed addition and mixing of reagents, wash liquids and the like used in conducting various assay protocols. As seen in **FIGURE 9A**, a single chamber is provided for capture and detection of the analyte of interest; **FIGURE 9B** shows an exploded view of a part of an alternative embodiment of the device having an analyte capture chamber and a separate analyte detection chamber; **FIGURE 9C** shows an exploded view of part of another embodiment of the device including a branched flow path region which permits analyte detection based on flow restriction in the branched region.

FIGURE 10A is a plan view of a diagrammatic representation of another embodiment of an analytical device for carrying out various assay protocols on microvolume samples, which may be used together with the sample preparation device of the present invention;

FIGURE 10B is an exploded fragmentary plan view of a part of the first flow passage through which sample fluid flows upon its introduction into the sample inlet port of the device shown in **Figure 10A**;

FIGURE 10C is a fragmentary transverse cross-section of the first flow passage taken along the line 10C-10C in **Figure 10B**, showing the side-by-side v-shape channels which constitute the first flow passage;

FIGURE 10D is a fragmentary longitudinal cross-section of the first flow passage taken along the line 10D-10D in **Figure 10C**, showing certain structural features of the barrier separating the v-shaped channels;

FIGURE 11A is a plan view of a diagrammatic representation of an analytical device intended for use with the sample preparation device of the invention, the analytical device having a series of mesoscale chambers suitable for implementing a variety of procedures including cell sorting, cell lysing and polynucleotide amplification, e.g., PCR; **FIGURE 11B** is a plan view of a diagrammatic illustration of an alternative design for a mesoscale PCR analytical device.

FIGURES 12A and 12B are fragmentary plan views of additional embodiments of microfabricated, restricted flow separators disposed in the flow path of a sample preparation device of the invention.

FIGURES 12C and 12D are fragmentary longitudinal sectional views of other additional embodiments of microfabricated restricted flow separators disposed in the flow path of the sample preparation device of the invention.

[0024] Like reference characters designate like parts in the drawing figures in which they appear.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The sample preparation device of the invention generally comprises a solid substrate, preferably in the form of a chip having dimensions on the order of less than one to a few millimeters thick and approximately 0.1 to 5.0 centimeters square. The substrate is microfabricated to form a sample flow path having an inlet and an outlet as well as a separator disposed intermediate to the inlet and outlet. The upstream-facing portion of the separator defines a separation zone in the flow path in which particulate components of the test sample are collected. The device also includes a flow channel in fluid communication with the separation zone which functions to discharge collected particulate components from the separation zone. The flow channel has an inlet section for directing a carrier fluid into the separation zone and over the upstream-facing portion of the separator and a discharge section for directing the carrier fluid, in which the particulate components are entrained, out of the separation zone. At least one of the aforementioned flow-path-and-flow channel sections have at least one mesoscale dimension.

[0026] If the particulate components of the sample are not to be analyzed, they can remain in the separation zone, in which case the flow channel is essentially nonfunctional and thus may be eliminated from the device.

[0027] As used herein, the term "mesoscale" refers to flow passages or channels and other structural elements, e.g. reaction and/or detection chambers, at least one of which has at least one cross-sectional dimension on the order of 0.1 μm to 1000 μm and more preferably 0.2 μm to 500 μm . The preferred depth of the flow passages and chambers is on the order of 0.1-100 μm and more preferably 2-50 μm . The preferred flow passage width is on the order of 2-200

μm and more preferably 3-100 μm . The preferred chamber width is on the order of 0.05-5 mm and more preferably 50-500 μm . The width of the passageway(s) in the separator is typically on the order of less than 50 μm which is sufficiently small to separate particulate matter from most biological samples and other test samples of interest. The separator passageways will normally have a depth of about 0.1 to about 100 μm . The length of the separator passageways will typically be within the range of about 0.1 μm to about 5 mm.

[0028] The flow passages and other structures, when viewed in cross-section, may be triangular, ellipsoidal, square, rectangular, circular or any other shape at least one cross-sectional dimension of which, transverse to the path of flow of sample fluid through or into a given structure, is mesoscale.

[0029] The mesoscale devices of the invention facilitate sample preparation in a broad range of biological analyses and, together with the analytical devices described herein, enable the rapid determination of microquantities of both molecular and cellular analytes in various test samples. At the conclusion of the analysis, the devices typically are discarded.

[0030] Mesoscale devices having at least one flow passage or other structural element with at least one mesoscale dimension can be designed and fabricated in large quantities from a solid substrate material using various micromachining methods known to those skilled in the art. Such methods include film deposition processes, such as spin coating and chemical vapor deposition, laser machining or photolithographic techniques, e.g. UV or X-ray processes, etching methods which may be performed by either wet chemical processes or plasma processes, LIGA processing or plastic molding. See, for example, Manz et al., Trends in Analytical Chemistry 10:144-149 (1991).

[0031] The sample preparation device of the invention may be conveniently constructed by forming the flow passages and separator in the surface of a suitable substrate and then mounting a cover over such surface. The solid substrate and/or cover may comprise a material such as silicon, polysilicon, silica glass, thermocouple materials, gallium arsenide, polyimide, silicon nitride and silicon dioxide. The cover and/or substrate may also comprise a plastic material, such as acrylic, polycarbonate, polystyrene, polyethylene or other resin materials. Optionally, the cover and/or substrate may comprise a transparent material, e.g., a relatively thin, anodically bonded layer of glass or ultrasonically welded plastic sheet material. Alternatively, two substrates of like material can be sandwiched together, or a suitable substrate material may be sandwiched between two transparent cover layers.

[0032] A diagrammatic representation of one embodiment the mesoscale sample preparation device of the invention is shown in FIGURE 1. The device 10 is microfabricated in a suitable substrate 11, thereby forming a sample flow path 12a and 12b having sample inlet port 14 and outlet port 16. A filter-type separator 18 is interposed in the flow path between inlet 14 and outlet 16. The upstream-facing portion 20 of the separator defines a separation zone 22 for collecting particulate components of the test sample. The device also includes a flow channel 24a and 24b in fluid communication with separation zone 22 for delivering a carrier fluid to, and discharging collected particulate matter from the separation zone. Flow channel 24a, 24b has an inlet section 26 for directing carrier fluid, e.g., isotonic buffer, from a source (not shown) over the upstream-facing portion 20 of separator 18. Discharge section 28 conveys the carrier fluid from over the upstream-facing surface of the filter element and out of separation zone 22.

[0033] Separator 18 which is microfabricated in sample flow path 12a and 12b of the sample preparation device serves to remove particulate matter from the test sample passed through the device prior to analysis. In one embodiment, shown in FIGURES 2 and 3, the separator comprises a series of mesoscale passageways of reduced dimension in comparison with flow path 12a, 12b. In operation, separator 18 functions as a filter, accumulating particulate matter on its upstream surface 18a, while the filtrate exiting passageways 19 continues along flow path 12b. The filter passageways 19 are microfabricated with depths and widths on the order of about 5 μm to about 50 μm , whereas flow paths 12a, 12b have maximum depths and widths on the order of approximately 1000 μm . The filter element is preferably microfabricated in the substrate of the device so as to form at least one, and preferably several, generally upstanding projections of the substrate material disposed in the flow path, which serve to restrict the flow of sample fluid through the separation zone.

[0034] Protuberances p may be provided on the exterior of the upstream-facing portion of separator 18, as depicted in Figure 2, as an aid in preventing plugging of passageways 19 by particulate matter in the sample fluid. Also, a sump (not shown) may be provided adjacent the upstream-facing portion of separator 18 for collecting insoluble debris removed from the sample fluid.

[0035] Separator 18 preferably is an essentially stationary structure permanently positioned between sample inlet 14 and outlet 16 of the flow path, as can be seen in FIGURE 1. Alternatively, however, the separator may be transiently disposed in the flow path. For example, a mass of magnetic particles may be retained in relatively fixed position in flow path 12a, 12b by means of an applied magnetic field to effect filtration of particulate matter from the test sample. The fluid portion of the sample passes through the void spaces between the particles as the filtrate. At the appropriate time, the applied magnetic field is removed and the magnetic particles may be transferred from the flow path, together with any particular matter from the test sample accumulated thereon, for analysis or disposal, as desired.

[0036] Separator 18 may, if desired, comprise a reagent that facilitates removal of particles or formed bodies from the test sample. In the case of a biological sample comprising a mixed cell population, for example, a binding substance

that releasably binds to a specific target cell type within the mixed population may be adsorbed or otherwise affixed to the separator to effect removal and selective retention of the target cell type. Cells which are not retained can be conveyed from the separation zone for disposal. The retained cells are subsequently caused to be released for analysis.

[0037] The sample preparation device of the invention can be used in combination with an appliance, such as appliance 30, shown in schematic cross-section in FIGURE 4, for delivering fluids to, discharging fluids from, and transferring fluids between the different devices constituting the analytical systems of the invention. Appliance 30, which has a nesting site 32 for holding the device 10, and for registering ports, e.g. port 14 on the device, with a flow line 33 in the appliance. The appliance may include an impellent, such as pump 34 shown in FIGURE 4, for conveying the sample through the flow passages of the device. After a biological fluid sample suspected to contain a particular analyte of interest is applied to the inlet port 35 of the appliance, pump 34 is actuated to convey the sample into port 14 of device 10 and then through flow path 12a,12b. Although pump 34 is shown as an element of appliance 30, it may, if desired be incorporated into device 10 according to known microfabrication techniques. Economic considerations, however, favor placement of the pump in appliance 30. Alternatively, depending on the nature of the analyses to be performed, a sample may be injected into the device, or the sample may enter the flow passages of the device through the inlet port by capillary action. In another embodiment, the appliance may be disposed over the sample preparation chip, and may be provided with a flow line communicating with the inlet port in the device, e.g., in the absence of a cover over the device, to allow a sample to be injected into the device. The microfabricated structures of the devices may be filled to a hydraulically full volume and the appliance may be utilized to direct the flow of fluid through the structures, e.g., by means of valves located in the appliance, the incorporation of valves in a microfabricated silicon chip can be accomplished according to techniques known in the art.

[0038] The outlet 36 of appliance 30 may be interconnected to the inlet of a similar appliance holding an analytical device of the type described herein, whereby the sample prepared in device 10 is transferred to the analytical device for testing.

[0039] The analytical devices also may be utilized in combination with an appliance for viewing the contents of the mesoscale flow passages and other structures in the devices. For example, the appliance may comprise a microscope (not shown) for viewing the contents of the mesoscale structure(s) in the device. Transparent cover 29, as shown in FIGURE 1, serves as a window which facilitates dynamic viewing of the contents of the device.

[0040] FIGURE 5 shows a diagrammatic representation of the combination of the sample preparation device of FIGURE 1 and analytical device 110 designed to carry out various binding assay protocols, and also polynucleotide amplification. To this end the device 110 is provided with an assay structure 112 and a polynucleotide amplification/assay structure 122. In the embodiment illustrated in FIGURE 5, the outlet of flow path 12a, 12b is in fluid communication with the inlet port 114 of assay structure 112 of the device; and the discharge section 28 of channel 24a, 24b is in fluid communication with the inlet port 124 of polynucleotide amplification/assay structure 122. Reagents used in performing the assay or other test or analysis may be introduced through reagent inlet ports 116 or 126, respectively. A reaction region 117 is typically provided in assay structure 112 in which a suitable reagent interacts with the analyte to yield a detectable product which is determinative of the analyte. That is to say, the product produced is one which provides definite information as to the nature or quantity of the analyte. The product may be detected in the form in which it is produced in reaction region 117, or it may be subject to further reaction to enhance its detection. A separate reaction/detection region 118 may be provided for this purpose.

[0041] A solution containing analyte-specific binding substances may be introduced into reaction region 117 via an inlet port (not shown) in fluid communication with the reaction region. Protein binding substances introduced in aqueous solution may be retained in a mesoscale structure in lyophilized form. Alternatively, binding substances may be immobilized in a mesoscale chamber of the analytical devices after its manufacture by, for example, physical adsorption or chemical attachment to the surface of the chamber or to a mobile, solid phase support, such as magnetic or non-magnetic polymer particles disposed in the chamber.

[0042] In carrying out polynucleotide amplification using device 110, cells of interest transferred from discharge section 28 of the sample preparation device 10 are subject to lysis either by a lysing agent or by a lysing structure as described in the above-mentioned U.S. Patent No. 5,304,487. The target polynucleotide released from the cells undergoes amplification in amplification region 127 and the amplified polynucleotide may be detected in detection region 128. One or more of the apertures 116, 119, 126 and 129 may be open to the atmosphere to vent the system(s). The operation of the binding assay structure 112 and the polynucleotide amplification/assay structure 122 will be further explained with reference to other embodiments of such devices described below.

[0043] Although assay structure 112 and polynucleotide amplification/assay structure 122 are fashioned on a common substrate as a single device, as shown in FIGURE 5, the structures may be fabricated on separate substrates and function as distinct analytical devices or chips, as will appear below.

[0044] When the sample preparation device and analytical devices described above are used together to function as a analytical system, as illustrated in Figure 5, for example, the system is advantageously combined with an appliance of the type depicted in Figure 6A, 6B and 7. Like the appliance of Figure 4, previously described, appliance 50 in Figure

6A serves to deliver fluid to, discharge fluid from, and transfer fluid between the respective devices. Appliance 50 has a nesting site 52 for holding sample preparation device 10 and analytical device 110 and for registering ports in the devices with flow lines in the appliance. Specifically, flow line 54a is in registry with inlet port 14 of the sample preparation device, flow line 54b is in registry both with outlet 16 of the sample preparation device and inlet 114, and flow line 54c is in registry with outlet 119 of assay structure 112 of the analytical device. As illustrated in Figure 6A, flow line 54a is in fluid communication with appliance inlet port 56, whereas flow line 54C is in fluid communication with appliance outlet 57. The appliance typically includes an impellent, such as pump 58, for forcing sample fluid through the analytical system. After applying to inlet port 56 of appliance 50, a particle-containing fluid test sample, e.g.; whole blood, the serum phase of which is suspected to contain an analyte of interest, pump 58 is actuated to force the sample through separator 18, providing sample fluid, e.g., serum, of substantially reduced particle content. The substantially particle-free sample fluid is transferred from device 10 via flow line 54B to assay structure 112 for testing, e.g., immunoassay.

[0045] The binding of analyte, *per se*, or analyte reaction products to a binding substance in the reaction/detection region of the analytical devices can be detected by any number of methods, including monitoring the pressure or electrical conductivity of sample fluids in the device(s), as disclosed in the above-referenced related applications (see, for example, U.S. Serial No. 877,702), or by optical detection through a transparent cover, either visually or by machine. For example, reaction of an analyte with a binding substance in the reaction region 117 of analytical device 112 illustrated in Figure 6A can be detected by monitoring the pressure of the sample fluids in certain regions of the mesoscale flow passages. This is accomplished in the analytical system-appliance combination of Figure 6A by means of two pressure detectors 59a and 59b for detecting flow pressure of fluids entering and exiting the devices through ports 14 and 119, respectively. When, during the performance of an assay, particles agglomerate or molecules chemically interact to form a network causing restricted flow or an increase in the viscosity of the sample liquid passing through the reaction/detection region, such changes can be detected as a pressure change which is indicative of a positive result. Mesoscale pressure sensors, and other electrical or electro-mechanical sensors can be directly fabricated on a silicon substrate and can be mass-produced according to well established techniques. Angell et al., *Scientific American*, 248: 44-55 (1983).

[0046] Other embodiments of appliances may be fabricated for use in carrying out different assay protocols with different devices in accordance with the present invention. One such embodiment is depicted in Figure 6B, which illustrates a cross sectional view of an analytical system, comprising analyte device 110' stacked upon a sample preparation device 10', disposed in nesting site 72 provided in appliance 70. A particle-containing test sample fluid is applied to appliance sample inlet 74, whereupon an impellent, such as pump 75, causes the sample fluid to pass through device 10', providing a sample fluid of substantially reduced particle content for analysis in analytical device 110'. The cover 116' of analytical device 110' has an aperture 114' open to the atmosphere to vent the system. Placement of the analytical device 110' on the top of the stack allows optical detection through a transparent portion of cover 116'.

[0047] A separate view of an analytical system, comprising a sample preparation chip and an analytical device for polynucleotide amplification, in combination with an appliance of the type described above is provided in Figure 7. The cross-sectional view of the analytical system in Figure 7 shows appliance 90 having a nesting site occupied by sample preparation device 10 and the polynucleotide amplification/assay structure 122. The discharge section 28 of flow channel 24b in sample preparation device 10 is in fluid communication, through flow line 92 with the inlet port 124 of polynucleotide amplification/assay structure 122. Flow line 93 is in registry with outlet 129 of the analytical device and in fluid communication with appliance outlet 94.

[0048] The polynucleotide sample, after release from the cell component separated from the sample fluid in sample preparation device 10, e.g., by contacting with the suitable lysing means as described above, is introduced into amplification region 127. Reagents required for amplification are also added to amplification region 127 through inlet 126, as shown in Figure 5. An impellent, such as a pump (not shown), is used to deliver the polynucleotide sample through flow line 92 to amplification region 127.

[0049] Amplification reagents may be similarly delivered to amplification region 127 through a different flow line provided in the appliance or in the analytical device (not shown). The product of the polynucleotide amplification reaction may be transferred to region 128 for detection in the manner previously described. The resultant product may be recovered, if desired, through appliance outlet 94.

[0050] Pressure differentials along the path of flow of the test sample fluid through devices 10 and 122 may be measured using pressure sensor 96 in conjunction with a pressure sensor (not shown) deployed in the appliance or the device to measure pressure at a point upstream of discharge section 28 of device 10.

[0051] Appliance 90 may include a heating/cooling element 95 for controlling the temperature within the polynucleotide amplification region, e.g., an electrical heating element and/or a refrigeration element. An electrical heating element (not shown) may alternatively be integrated into the substrate of analytical device 122, with electrical elements for power mated to matching electrical contacts in the appliance below the amplification region 127. Alternatively, the appliance may include an internal or external heating means, such as a laser or other source of electromagnetic energy (not shown) disposed adjacent amplification region 127 of polynucleotide amplification/assay structure 122. A micro-

processor in appliance 90 may be used to regulate the heating element in order to provide a temperature cycle in the polynucleotide amplification region between a temperature suitable for dehybridization, e.g., 94°C, and temperatures suitable for annealing and polymerization, e.g., 65°C. A thermocouple may also be provided in the substrate surrounding amplification region 127 in electrical contact with the appliance to allow microprocessor or other electronic controller to detect and maintain the temperature cycles in the reaction chamber. A cooling element, such as a miniature thermoelectric heat pump (Materials Electronic Products Corp., Trenton, NJ), may also be included in the appliance for adjusting the temperature of the amplification chamber. In another embodiment, the temperature of the polynucleotide amplification chamber can be regulated by a timed laser pulse directed at the reaction chamber through glass cover 109, so as to allow sequential heating and cooling of the sample to the required temperatures for the amplification cycle. The thermal properties of silicon enable a rapid heating and cooling cycle.

5 [0052] In all of the embodiments of the invention depicted in Figure 4, 6A, 6B and 7, the pump may be subject to control by a microprocessor in the appliance. Also, the devices illustrated in the last-mentioned figures may be retained securely engaged in the nesting site of the appliance, or in contact with one another, as the case may be, in various ways including, by way of example, a clamp (not shown) mounted on the appliance, binding of the confronting device surfaces to one another, e.g., by adhesive, or by appropriate dimensioning the devices relative to the nesting sites to frictionally retain the devices therein.

10 [0053] A biological assay device which may be used in combination with the sample preparation device of the invention is shown in FIGURE 8A. The device 130 was fabricated on a substrate 131 having mesoscale flow channels 132a, 132b with entry ports 133 microfabricated on opposite ends of the channels and a central mesoscale mixing/capture/detection chamber 135. As depicted in FIGURE 8A, the cross-sectional dimension of chamber 135 is relatively larger than that of channel 132a, 132b.

15 [0054] A capture reagent, such as a substance that binds specifically to the analyte of interest, may be immobilized, either on a stationary or mobile support, in chamber 135. When a mobile support, e.g. polymer particles, is used, the particle size should be selected so as to be relatively larger than the cross-sectional dimension of flow channel 132a, 20 132b in order that the immobilized reagent is confined to chamber 135. A reagent immobilized on a particulate solid support in this manner can conveniently be charged to chamber 135 via inlet port 137.

25 [0055] A device of the type just described can be used to carry out various immunoassay reactions. For example, a non-competitive, immunometric assay for the determination of carcinoembryonic antigen (CEA) may be carried out by filling chamber 135 with monoclonal anti-CEA antibodies immobilized on a particulate support, such as plastic beads. 30 The test sample to be analyzed for CEA is then added to fill chamber 135 and expel any fluid introduced with the immobilized reagent. The contents of chamber 135 are thereafter incubated for a time sufficient to effect antigen-antibody binding. Subsequently, an antibody enzyme conjugate, e.g. monoclonal anti-CEA antibody-horseradish peroxidase is added to the chamber and the contents are again incubated. A solution of a chromogenic substrate is then added to chamber 135 which serves to wash the immobilized reagent, expelling unbound conjugate. Sufficient substrate is retained in the chamber to react with any peroxidase label bound to the immobilized reagent. The rate of generation 35 of chromophore is directly proportional to the concentration of CEA in the sample.

40 [0056] Device 130 may also be used to perform a competitive assay for the determination of thyroxine in a test sample. In carrying out this format, chamber 135 is filled with an immobilized reagent comprising anti-thyroxine antibodies bound to the surface of plastic beads. The test sample to be analyzed for thyroxine is premixed with a thyroxine-peroxidase conjugate and added to the chamber, thus filling the chamber and expelling any fluid introduced with the immobilized reagent. The contents of the chamber are then incubated for a time sufficient to effect antigen-antibody binding. A buffer may optionally be passed through chamber 135 to wash the immobilized reagent. A chromogenic substrate is thereafter added to the chamber, washing the immobilized reagent and expelling any unbound reagents. Sufficient substrate is retained in chamber 135 to react with any peroxidase label bound to the immobilized reagent. 45 Generation of chromophore is inversely proportional to the concentration of thyroxine in the test sample.

[0057] Although the assay structure of FIGURE 8A is configured to confine the immobilized reagent in channel 135, the design is such that fluid can be pumped over and through the immobilized reagent for washing purposes.

[0058] It should be understood that the last-mentioned two examples are merely representative, as the device of FIGURE 8A, as well as the other devices described herein may be used to implement a variety of other assay formats.

50 [0059] FIGURE 8B shows analytical device 140 microfabricated on a substrate 141 and having an inlet port 143 in fluid communication with a chamber 145 for analyte capture, e.g., by immunocapture. This device is adapted for carrying out enzyme immunoassay. To that end, the device includes a separate chamber 147 containing a binding agent to capture and concentrate the chromophore produced by the action of the enzyme label on a suitable substrate. For example, a protein analyte may be determined using a "sandwich" assay technique, in which the analyte is captured 55 in chamber 145 by an antibody immobilized therein which binds specifically to the analyte. The captured analyte is labelled with an enzyme-antibody conjugate composed of alkaline phosphatase, for example, and an antibody that specifically binds the protein analyte. Fluorescein phosphate is introduced into chamber 145 as a chromogenic substrate for the enzyme label. Alkaline phosphatase acts on the substrate to generate fluorescein which is captured by

an anti-fluorescein antibody immobilized in chamber 147. A hydrophobic environment created in chamber 147, e.g., by virtue of material adhered to the walls of the structure, the capture agent or a component of the reaction mixture, e.g., a surfactant or micelle-forming agent, will improve the fluorescent signal from the bound fluorescein. Detection of the chromophore may be carried out in chamber 147 or the chromophore may be removed from the device through outlet 149 for detection in a separate apparatus. other substrates could be selected for use in carrying out this determination, such as 4-nitrophenol phosphate or 4-methylumbelliflone phosphate, with appropriate binding agents used to capture the dephosphorylated product.

[0060] A diagrammatic representation of another embodiment of a biological assay device that may be used in the practice of the present invention is shown in FIGURE 9. The substrate 151 of device 150 is microfabricated with ports 152a-e, flow channels 154a-g, reaction chambers 156a and 156b and a capture/detection chamber 158. The reaction chambers 156a and 156b each comprise a tortuous mesoscale flow channel. The path length of the tortuous channel may be designed to permit the timed mixing and addition of sample reagent(s). Devices of this type may be utilized in combination with an appliance having ports mated to ports in the device, which appliance is capable of delivering and receiving fluids through the flow system of the device and, optionally, capable of optically detecting a positive or quantitative result in chamber 158. In one application of the device, the cholesterol content of a sample may be determined. Cholesterol esterase is applied via inlet port 152a and buffer and sample are added via inlet ports 152b and 152c, respectively. The mixture then flows through channel 154d to the tortuous mixing/reaction chamber 156a. The time of mixing and reaction may be predetermined by microfabricating the tortuous channel to the appropriate length and controlling the flow rates. Cholesterol oxidase is added via port 152d and flows through channel 154g to the tortuous channel 156b where the timed mixing and reaction of the cholesterol oxidase with the fluid from channel 156a occurs. Heating means like those described above, may be provided to maintain the device at 37°C, or higher. A chromogenic substance is introduced at 154e through a flow channel (not shown) for detection. Positive or quantitative results can be detected optically by observing the detection chamber 158, e.g., through an optical window disposed over the chamber.. The detection chamber 158 may be provided with an immobilized binding moiety capable of capturing the product of the enzyme reaction, thus facilitating detection. This device may be applied to a range of clinical enzymatic and other reactions.

[0061] According to an alternative embodiment shown in FIGURE 9B, capture of a fluorescently labelled analyte may occur in chamber 158a, which contains an analyte-specific binding agent that binds releasably to the analyte. Released fluorescently labelled analyte is captured for detection in chamber 158b.

[0062] In another embodiment illustrated in FIGURE 9C, flow channel 154f may be constricted, such that the flow path is of smaller cross-sectional area than channel 154e, thereby restricting flow of test fluid through the device. As depicted in FIGURE 9C, channel 154f is constructed in a pattern of parallel flow channels, with reduced dimensions at each channel division, providing sequentially narrower flow passages. This device may be utilized in performing various agglutination assays, the occurrence of particle-induced or complex-induced agglutination being detected on the basis of restricted flow of the sample through the branched portion 159 of flow channel 154f.

[0063] FIGURE 10A is a diagrammatic representation of a mesoscale analytical device 170 design for carrying out various binding assay protocols. The device enables determination of a range of analytes on the basis of microvolumes of sample and small, measured amounts of reagents, with labelled product being detected within the device, so that all sample, unreacted reagent and reaction products remain confined in the device for subsequent disposal.

[0064] The device may be used in combination with an appliance (not shown) of the general type described above with reference to Figure 6A. Such a device has a nesting site for holding the device, flow lines and associated pumps and valves for delivering sample, reagents, wash solutions and the like to the device. The appliance may also include a temperature control and sensing means, pressure sensors and/or electrical connections to facilitate analyte detection, optical detection means, signal amplification and quantitation means, all as described herein. The combination may also include overall system sequence and control elements, quantitated information display and recording means via a microprocessor in the appliance, for example, or by interfacing with an external computer.

[0065] The device is microfabricated as previously described with the flow passages configured to provide a total capacity in the range of 0.01-100 µL, preferably from about 0.5 to about 50 µL.

[0066] In use, a microvolume of test sample fluid is introduced at port 171. The test sample fluid is pre-filtered by passage through the sample preparation device of the invention, before introduction at port 171. Alternatively, the sample fluid may be filtered after introduction into device 170. Internal filtration may be beneficially achieved by a cross-flow-filtration-technique.-As shown in Figure 10B, flow-passage 172, through-which sample-fluid initially-passes upon introduction at inlet 171, is divided into two side-by-side V-shaped channels 172a and 172b, separated by a longitudinal barrier 173, which is preferably formed from the substrate material (but may be a part of, and suspended from the cover plate or sheet). Barrier 173, together with the cover of the device, defines at least one passageway 174, as illustrated in Figure 10C, which allows fluid flow therethrough, but is of sufficiently small dimension to prevent the passage of particulate components, e.g., cells, of a fluid sample. Barrier 173 is positioned such that inlet 171 feeds sample fluid directly into flow path 172a and indirectly into flow path 172b, the fluid passing into flow path 172b having

a substantially reduced particle content, as compared with previously unfiltered sample entering inlet 171.

[0067] Flow passage 172 may be fabricated with walls that diverge from a relatively small cross-sectional dimension to a relatively larger cross-sectional dimension in the downstream direction from the inlet, or with walls that converge from a relatively large cross-sectional dimension to a relatively smaller cross-section dimension in the downstream direction from the inlet, with barrier 173 being disposed generally parallel to at least one of the passage walls. Such design gives rise to nonlinear flow of the sample fluid which aids in dislodging particles from passageway 174.

[0068] If the test sample fluid is filtered externally to device 170, the above-described internal filter may be omitted. Alternatively, a sample fluid that has been externally filtered can be entered directly into the device via port 175, thus bypassing flow passage 172. A buffer may also be introduced through port 175 for the preparation of diluted sample fluid, if desired. Excess buffer may be collected in outlet 176.

[0069] Particulate matter trapped in flow path 172a is conveyed to outlet 176, as illustrated in Figure 10B.

[0070] Filtrate from flow path 172b next passes into flow passage 177 which is appropriately dimensioned to function as a metering chamber, providing a pre-determined sample volume for analysis. The pre-determined sample volume will ordinarily be on the order of about 1 μL . A scale 178 may be provided on device 170, e.g., by etching, to aid in the metering of desired amounts of sample fluid into the device for analysis. By enabling the introduction of prescribed sample volumes into device 170, flow passage 177 also permits quantitation of the analyte.

[0071] A suitable impellent (not shown) incorporated in device 170, or in an appliance designed for use in conjunction with such device, can be employed for transferring the metered sample fluid to flow passage 179, which is optionally provided for mixing the sample fluid with the primary reagent used in performing the binding assay. The inclusion of such a mixing chamber in device 170 is beneficial for achieving more rapid and complete reaction between analyte and primary reagents.

[0072] Suitable impellents for transferring sample fluid, reagents, buffers and the like through the flow system of device 170 includes various pumps, such as micromachined pumps, diaphragm pumps, syringe pumps, volume occlusion pumps, as well as endosmotic induced flow, flow induced by electrochemical evolution of gases and other pumping means known to those skilled in the art.

[0073] The primary reagents may be delivered directly to flow passage 179 in the device through inlet 180. The primary reagents are caused to mix with the metered sample fluid upon entering flow passage 179, which may be sequential or essentially simultaneous. Excess primary reagents may pass out of the flow system through outlet 181.

[0074] The source of primary reagent may be an internal storage chamber which can optionally be provided in device 170. Alternatively, the primary reagents can be delivered to the device from a reservoir in an appliance with which the assay device is used, such as the appliance described with reference to Figure 6A, above, or from some other source external to the device. The primary reagents can be stored as liquid solutions, gels or neat, such as in dried or lyophilized form, or in any other convenient form. For example, the primary reagent can be lyophilized in place in flow passage 179, in which case the test sample fluid or a suitable solvent introduced, for example, through inlet 180 can be used to dissolve the primary reagents. Alternatively, the test sample or a solvent may be directed by liquid transfer means, as noted above, from flow channel 179 to a storage chamber (not shown) outside the flow system illustrated in Figure 10 to dissolve the primary reagents. In addition, heating or agitation means (not shown) may be provided in the storage chamber to aid in dissolving the primary reagents stored therein.

[0075] The primary reaction mixture, comprising the sample fluid and dissolved primary reagents can also be reacted in flow channel 179, which may include structural elements, as previously described, to promote turbulent flow. Agitation or other means may be provided to ensure adequate mixing of the primary reaction mixture. The primary reaction mixture is caused to remain in flow channel 179 for a time sufficient for the desired reaction to proceed to completion.

[0076] Means for regulating the temperature in flow channel 179, such as that previously described with reference to Figure 7, may optionally be utilized to enhance the primary reaction conditions. Means for sensing the temperature in flow passage 179 may also be provided, if desired. The temperature sensing means may be operatively connected to a microprocessor or similar device which controls the overall function of the system so as to correlate the sensed temperature with the residence time of the primary reaction mixture in flow passage 179.

[0077] Upon completion of reaction, all or part of the primary reaction mixture can be transferred, e.g., by the above-described pumps or other impellents, to capture region 182 and detection region 183, in which one or more original components of the sample fluid or products of the primary reaction may be monitored and/or detected. Alternatively, the product of a secondary reaction, the existence or concentration of which is correlatable to the existence or concentration of the analyte of interest in the sample fluid, can be employed for analyte determination.

[0078] The detection techniques utilized in connection with device 170 are those customarily used in performing binding assays. Briefly, these include chemical tests, such as may be carried out by addition of test reagents; spectroscopy, for example, to detect changes in properties of the analyte caused by chemical changes during the primary reaction, such as shifts in absorbance, wave lengths, changes in fluorescence polarization, changes in fluorescence stokes shifts, and the like; agglutination, as measured by microscope, image analysis or similar procedures; and measuring electrochemical performance of the reacted primary reaction mixture, such as specific measurement by amper-

ometric and/or potentiometric/voltametric techniques.

[0079] With regard to carrying out a secondary reaction for analyte determination, a capture region, defined by flow passage 182, is provided into which all or part of the reacted primary reaction mixture is transferred by liquid transfer means of the type previously described, and in which one or more components of the products in the primary reaction mixture may be captured by binding to a surface and subsequently detected and/or quantitated. Capture reagent may be immobilized on the walls of flow passage 182 or on the surface of particles or beads present in flow passage 182, or both.

[0080] An inlet or fill hole 184 may be provided to pre-fill flow passage 182 with solid phase capture reagent comprising plastic, latex, silica or other suitable support material, including magnetic components, capable of combining specifically to the products of the primary reaction mixture. The particulate capture reagent can be charged to flow passage 182 either as a wet slurry, which may subsequently be dried or lyophilized, or in dry form. In either case, the filling of flow passage 182 can optionally be assisted by vibration or other means. The mobile solid phase of the capture reagent comprises particles or beads having diameters from tens of nanometers to tens of microns, with a surface coating of avidin, streptavidin or other substance to which biotinylated or otherwise conjugated antibodies will specifically bind.

[0081] Flow passage 182 may be fabricated with flow restricting structural elements 189a, 189b or other means to confine the capture reagent within flow passage 182 while allowing passage of fluids therethrough. The particulate capture reagent may also be confined within flow passage 182 in the manner previously described with reference to Figure 8A.

[0082] The primary reaction mixture is caused to remain in flow passage 182 for a time sufficient for reaction with the capture reagent to proceed to a known extent, preferably essentially to completion. Means for regulating and sensing the temperature in flow passage 182 may optionally be provided as noted above with reference to flow passage 179.

[0083] The captured product of the primary reaction mixture is preferably washed before proceeding with the secondary reaction.

[0084] The reagent solution for the secondary reaction may be delivered directly to device 170 via inlet 185. Excess secondary reagent may be removed from the flow system through outlet 186 or 187. Alternatively, the reagent for the secondary reaction may be kept prior to dissolution and use in a storage chamber in device 170, or in an appliance used in conjunction with the device, or in some other convenient source external to the device. One or more flow lines appropriately mated with flow passages in device 170 and operatively connected to an impellent may optionally be provided to transfer solvent from an input port to the above-mentioned secondary storage chamber where stored reagents are dissolved to form the secondary reaction solution.

[0085] The reagent for the secondary reaction may include an enzyme substrate specific to an enzyme conjugated to the captured primary reaction product, as well as substances which, when dissolved in the secondary reaction solution, assist in washing of the bound primary reaction product.

[0086] The secondary reaction preferably occurs in flow passage 182, wherein the secondary reaction solution reacts with captured primary reaction products. The product of the secondary reaction may be a substance selected from the group of molecules or ions directly or indirectly detectable based on light absorbance, fluorescence, phosphorescence properties; molecules or ions detectable by their radioactive properties; or molecules or ions detectable by their nuclear magnetic resonance or paramagnetic properties. The product of the secondary reaction may be amplified, according to procedures known in the art to enhance the detection thereof. For example, an enzyme amplification reaction may be employed, which releases a fluorophore generated from a non-fluorescent precursor in the secondary reaction solution.

[0087] After the secondary reaction is complete, the resultant product may be detected and quantitated either within flow passage 182 or subsequently in detection region 183, or in a detector external to device 170.

[0088] The preferred cross-sectional dimensions of flow passages 177 and 183, transverse to the path of flow of sample fluid, are about 100 µm wide and 70 µm deep, whereas the preferred cross-sectional dimensions of flow passages 179 and 182, transverse to the path of flow of sample fluid, are about 400 µm wide and 70 µm deep. These dimensions are within the mesoscale range, as set forth above.

[0089] Various binding assay protocols can be implemented in device 170 including immunometric (sandwich) assays as well as competitive immunoassays, employing both polyclonal and monoclonal antibodies for purposes of capture and detection of analyte. One form of detection antibody comprises a conjugated label wherein the label is fluorophore detectable as a bound moiety after capture on a solid phase. Another form of detection antibody comprises a conjugated label wherein the label is fluorophore detectable after release from the captured primary reaction product. Another form of detection antibody comprises a conjugated enzyme moiety such as horseradish peroxidase or alkaline phosphatase.

[0090] Washing steps may be carried out as appropriate to eliminate potentially interfering substances from device 170.

[0091] Excess sample fluid, reagents, wash solutions and the like from the various flow passages and structural elements may be combined and routed into a single waste receptacle of adequate capacity, preferably within device 170, such that all sample fluid and reaction products are safely contained for disposal.

[0092] FIGURE 11A diagrammatically depicts an analytical device 191 used to determine the presence of an intracellular polynucleotide in a biological cell-containing fluid sample, and then to perform an assay for a particular nucleotide sequence. Microfabricated on substrate 192 is a mesoscale flow path 194a-c which includes a cell separation chamber 196a, a cell lysis chamber 196b, a filter element 197, a polynucleotide amplification chamber comprising sections 198a and 198b, and a detection region 199. The mesoscale flow system is also provided with fluid entry/exit ports 193a-d. The device can be used in combination with an appliance, such as that described above with reference to FIGURE 6A.

[0093] Initially, the valves in the above-mentioned appliance function to close ports 193c and 193d, while ports 193a and 193b are open. A sample containing a mixture of cells, e.g., transferred from the sample preparation device, is directed to the sample inlet port 193a by a suitable impellent, e.g. a pump, (not shown), and flows through the mesoscale flow channel 194a to separation chamber 196a. Chamber 196a contains binding moieties immobilized on the wall of the chamber which selectively bind to a surface molecule on a desired cell type in the sample. Remaining cellular components exit the substrate via port 193b. After binding of the desired cell type in chamber 196a, flow with buffer is continued, to wash and assure isolation of the target cells. Next port 193b is closed and 193c is opened. Flow is then increased sufficiently to dislodge the immobilized cells from chamber 196a. Flow is continued, forcing cells through membrane piercing protrusions 195 in chamber 196b, which tear open the cells releasing intracellular material.

[0094] Sample flow continues past filter 197, which filters off large cellular membrane components and other debris, with the filtrate passing to mesoscale PCR chamber section 198a, which is connected to PCR chamber section 198b by flow channel 194b. Taq polymerase, primers and other reagents required for the PCR assay next are added to section 198b through port 193c from a source thereof (not shown), permitting mixing of the intracellular soluble components from the separated subpopulation of cells and the PCR reagents. With the ports closed (to ensure that the reaction mixture does not evaporate, or otherwise becomes lost from the device), an impellent, e.g. a pump, (not shown), applies a motive force to port 193b to cycle the PCR sample and reagents through flow channel 194b between sections 198a and 198b, set at 94°C and 65°C, respectively, to implement plural polynucleotide melting and polymerization cycles, allowing the amplification of the polynucleotide of interest. Before the next process step, port 193c is closed and port 193d is opened. The same impellent force is then used to direct the amplified polynucleotide isolated from the cell population to a detection region 199 in the form of a pattern of flow channels like that described above with reference to FIGURE 9C. Flow reduction in the restricted region serves as a positive indicator of the presence of amplified polynucleotide product and may be detected optically through a glass cover disposed over the detection region 199. Alternatively, the amplified polynucleotide product may be detected directly in the reaction chamber, using commercially available reagents developed for such purpose, such as the "Taq Man®" reagents, available from Perkin Elmer Corporation. The amplified polynucleotide may also be detected outside the device using various methods known in the art, such as electrophoresis in agarose gel in the presence of ethidium bromide.

[0095] Another embodiment of an analytical device which is useful in the practice of this invention is illustrated in FIGURE 11B. The device 210 comprises a substrate 214 microfabricated with a mesoscale polynucleotide amplification chamber 222A. The device 210 can be used in combination with an appliance like appliance 90 shown in FIGURE 7. The appliance is provided with flow paths mated to ports 216A, 216B, 216C and 216D in device 210. The appliance may also include valves that allow the ports 216A, 216B, 216C and 216D to be mechanically opened and closed. In one embodiment, the flow system of the devices may be maintained at a hydraulically full volume, and valves in the appliance may be utilized to direct fluid flow. Chamber 222A is heated and cooled to temperatures appropriate to provide a dehybridization temperature, and annealing and polymerization temperatures, as required for PCR. Temperature of the reaction region can be controlled as previously described with reference to FIGURE 7.

[0096] The flow system illustrated in Figure 11B includes filter elements 224, of the general type described herein, to remove from the sample fluid filterable components having a tendency to interfere with the analysis.

[0097] In operation, a sample containing polymerase enzyme and other reagents required for PCR is delivered through inlet port 216A to reaction chamber 222A. With the ports closed, a heating element is then utilized to thermally cycle the reaction chamber between a temperature suitable for dehybridization and temperatures suitable for annealing and polymerization. When the PCR reaction cycle is terminated, ports 216B and 216D are opened, driving the contents of chamber 222A to detection region 222B, which region contains a polynucleotide probe, e.g., immobilized upon beads 292. A positive assay for the polynucleotide is indicated by agglutination of the beads in the detection region.

[0098] Although polynucleotide amplification has been described herein with particular reference to PCR, it will be appreciated by those skilled-in-the-art that the devices and systems of the present invention may be utilized equally effectively for a variety of other polynucleotide amplification reactions. Such additional reactions may be thermally dependent, such as the polymerase chain reaction, or they may be carried out at a single temperature (e.g., nucleic acid sequenced-based amplification (NASBA)). Moreover, such reactions may employ a wide variety of amplification reagents and enzymes, including DNA ligase, T7 RNA polymerase and/or reverse transcriptase, among others. Additionally, denaturation of polynucleotides can be accomplished by known chemical or physical methods, alone or combined with temperature change. Polynucleotide amplification reactions that may be practiced in the device of the in-

vention include, but are not limited to: (1) target polynucleotide amplification methods such as self-sustained sequence replication (3SR) and strand-displacement amplification (SDA); (2) methods based on amplification of a signal attached to the target polynucleotide, such as "branched chain" DNA amplification (Chiron Corp., Emeryville, CA); (3) methods based on amplification or probe DNA, such as ligase chain reaction (LCR) and QB replicase amplification (QBR); (4) transcription-based methods, such as ligation activated transcription (NASBA) ; and (5) various other amplification methods, such as repair chain reaction (RCR) and cycling probe reaction (CPR) (for a summary of these methods and their commercial sources, see pp. 2-7 of The Genesis Report, DX, Vol. 3, No. 4, Feb. 1994; Genesis Group, Montclair, NJ).

[0099] The sample preparation device of the invention may be used in conjunction with Mesoscale Polynucleotide Amplification Devices, which is the subject matter of U.S. Serial No. 08/308,199.

[0100] Briefly, the last-mentioned patent application relates to mesoscale devices for amplification of a preselected polynucleotide in a sample fluid. The devices are provided with a substrate microfabricated to include a polynucleotide amplification reaction chamber having at least one cross-sectional dimension of about 0.1 to 1000 μm .

The device also includes at least one port in fluid communication with the reaction chamber, for introducing a sample to the chamber, for venting the chamber when necessary, and, optionally, for removing products or waste material from the device. The reaction chamber may be provided with reagents required for amplification of a preselected polynucleotide. The device also may include means for thermally regulating the contents of the reaction chamber, to amplify a preselected polynucleotide. Preferably, the reaction chamber is fabricated with a high surface to volume ratio, to facilitate thermal regulation. The amplification reaction chamber also may contain a composition which diminishes inhibition of the amplification reaction by material comprising a wall of the reaction chamber, when such treatment is required.

[0101] Appliances 30, 50, 70 and 90, as shown in Figures 4, 6A, 6B and 7, respectively, may also be utilized to deliver metered amounts of sample, reagent buffer and the like, as well as to implement the timed addition of sample or other fluids to the devices in connection with the performance of prescribed analytical protocols.

[0102] In those cases where a microprocessor is included in the appliance it may be used to assist in the collection of data for one or a series of analyses.

[0103] Although analyte determination has been described above with particular reference to whole blood as the sample fluid, the analyte of interest may be present in test samples or specimens of varying origin, including other biological fluids such as whole blood containing anti-coagulants, dilute whole blood, lysed whole blood, whole blood containing assay reagents, serum, plasma, urine, sperm, cerebrospinal fluid, amniotic fluid, lavage fluids, tissue extracts, cell suspensions and any other sample fluid that can be beneficially analyzed using the device and systems described herein.

[0104] Figures 12A-D illustrate various additional embodiments of microfabricated, restricted flow separators which may be disposed in the flow passages of the devices described herein. The separator in Figure 12A is in the form of a plurality of partitions 251, projecting from opposite surfaces 252a, 252b of channel 253, so as to define a series of passageways 254a, 254b, which are aligned longitudinally along the channel. One or more intermediate partitions 255, projecting from the bottom of channel 250 may be disposed adjacent the downstream-facing portion of one or more of partitions 251, to stand as barriers or baffles within the flow path provided by aligned passageways 253.

[0105] Sample fluid passing through the relatively narrow passageways 254a, 254b at relatively high speed will tend to disperse into the space between consecutive partitions, while reducing in speed and moving into the dead volume corners of such space. When sample fluid then passes into the next successive inter-partition space, particulate matter may be relatively retained in the dead volume. Thus, for each passage into a subsequent inter-partition space, particulate matter is progressively retained and sample fluid becomes gradually more purified as it flows downstream through the partitions. with a sufficient number of partitions in series, progressive reduction in particle concentration would be enabled, the efficiency of which could be predetermined. Baffles 255 would assist in directing the sample fluid into the dead volume region.

[0106] In Figure 12C, there is shown a weir-type separator structure formed by barriers 257 projecting up from the bottom 258 of channel 250.

[0107] The separator structure shown in Figures 12C and 12D takes advantage of the propensity of particles to fall under the influence of gravity. This may be particularly useful in the analysis of whole blood, by promoting the sedimentation of erythrocytes. The sample fluid passes at high speed over barrier 257, then immediately slows. Any particulate matter falling towards the floor of channel 250 will experience a lower supporting velocity and a diminished opportunity of being swirled up over the next succeeding barrier. Passage of sample fluid over a series of such barriers may progressively reduce particulate concentration and produce gradually more purified sample fluid. One or more lips 259 suspended from cover plate 260 assists in downwardly directing the sample fluid.

[0108] The following examples are provided to describe the use of an analytical device to which the sample preparation device of the present invention may be attached.

EXAMPLE 1

[0109] A plastic-silicon composite assay device was fabricated by attaching a plastic (3M transparency sheet) cover over a silicon substrate 131, microfabricated with flow channels 132a, 132b having entry ports 133 on opposite sides of the channel and a central reaction/detection chamber 135, as shown schematically in Figure 8A. A dilution of anti-A (in 0.05 M sodium bicarbonate pH 9.6) and a 1:10 dilution of Type A blood in saline were introduced via syringe using a holder into the entry ports 133 on opposite ends of the channel 132a, 132b. The solutions mixed together in the central chamber 135 and agglutination was observed through the plastic cover by light microscopy. The results are summarized in the following table.

| ANTI-A | DILUTION | AGGLUTINATION IN CHANNEL |
|-----------------------|----------|--------------------------|
| Gamma Kit | 1:20 | + |
| Gamma Murine Mono | 1:20 | + |
| Gamma Human Dilution | 1:5 | + |
| Immucor Affinity pure | 1:100 | + |
| Immucor Ascites | 1:100 | + |

EXAMPLE 2

[0110] A solution of mouse IgG (50 µg/mL in 0.05 M sodium bicarbonate pH 9.6) (SIGMA Cat. No. 1-5381) and a 1:20 dilution of goat anti-mouse IgG (H&L) - fluorescence carboxylate beads (Polysciences, Inc.) in PBS buffer were introduced via syringe using a holder into the entry ports on opposite ends of channels 132a, 132b in another assay device prepared as described in Example 1. The solutions were mixed together in the reaction/detection chamber 135 and agglutination was observed through the transparent plastic cover by light microscopy.

Claims

1. A device for preparing a test sample, comprising particulate components, for analysis, said device comprising a sample flow passage having a sample inlet and an outlet in fluid communication and a separator disposed between said inlet and said outlet, said separator having an upstream-facing portion defining a separation zone in said flow passage in which said particulate components are collected, and a flow channel in fluid communication with said separation zone for affording discharge of collected particulate components from said separation zone, said channel having an inlet section for directing a carrier fluid into said separation zone, and a discharge section for directing said carrier fluid from over the upstream facing portion of said separator and out of said separation zone, at least one of said flow passage and said flow channel sections having at least one mesoscale dimension, said mesoscale dimension being a cross-sectional dimension of width or depth which is between 0.1 and 1000 µm, **characterised in that** the device does not include valves, and **characterised in that** said inlet section of said flow channel is arranged to direct said carrier fluid into said separation zone over said upstream-facing portion of said separator.
2. The device of claim 1, wherein said flow passage has at least one mesoscale dimension and said separator comprises a region of restricted flow in said flow passage, said region of restricted flow being formed by at least one passageway having at least one mesoscale dimension, which is smaller than the least mesoscale dimension of said flow passage and being sufficiently small to separate said particulate components from said test sample, both said mesoscale dimensions being a cross-sectional dimension of width or depth which is between 0.1 and 1000 µm.
3. The device of claim 2, wherein said at least one passageway has at least one bend therein such that at least a part of said passageway is generally perpendicular to said flow passage.
4. The device of claim 1, wherein said flow passage and said flow channel are formed in a surface of a solid substrate and enclosed by a cover adhered to said surface.
5. The device of claim 4, wherein said separator is in the form of at least one generally upstanding projection of said substrate which is disposed in said flow passage and which restricts the flow of test sample along said flow passage.
6. The device of claim 4, wherein said cover is transparent.

7. In combination, the device of claim 1 and an appliance for use with said device, said appliance comprising a holder for said device, a test sample input conduit interfitted with the sample inlet of said device and an impellant for moving test sample along said flow passage.
- 5 8. The combination of claim 7, wherein said appliance further comprises a reservoir for said test sample.
9. The combination of claim 7, wherein said appliance further comprises a carrier fluid input conduit interfitted with said inlet section of said flow channel and an impellant for moving carrier fluid along said flow channel.
- 10 10. The combination of claim 7, wherein said appliance further comprises a reservoir for said carrier fluid.
11. A system for determining an analyte in a fluid sample, said system comprising a device according to claim 1 and an analyte detection device wherein said analyte detection device comprises:
 - 15 a solid substrate fabricated to define:
 - a sample inlet port in fluid communication with the sample outlet of the device of claim 1; and a flow system comprising:
 - 20 an analyte detection region in fluid communication with said sample inlet port, said region containing a reagent which interacts with said analyte to yield a detectable product which is determinative of said analyte, and a detector for detecting said product.
 - 25 12. The system of claim 11, which further comprises, in said analyte detection device, a sample flow channel interconnecting said inlet port and said analyte detection region, at least one of said analyte detection region and said sample flow channel having at least one mesoscale dimension, said mesoscale dimension being a cross-sectional dimension of width or depth which is between 0.1 and 1000 µm.
 - 30 13. The system of claim 12, wherein said reagent is a binding substance that binds specifically to said analyte.
 14. The system of claim 13, wherein said analyte is an antigen and said binding substance is an antibody.
 15. The system of claim 13, wherein said analyte is a ligand and said binding substance is a receptor.
 - 35 16. The system of claim 13, wherein said analyte is a nucleic acid molecule of predetermined sequence and said binding substance is a nucleic acid molecule having a sequence complementary or homologous to the sequence of said analyte.
 - 40 17. The system of claim 11 further comprising a device for performing analysis of preselected polynucleotide derived from cells, said analysis comprising a polynucleotide amplification reaction, said polynucleotide analysis device comprising:
 - 45 a solid substrate fabricated to define:
 - a sample inlet port in fluid communication with the sample outlet of the device of claim 1; and a flow system comprising:
 - 50 a polynucleotide amplification region in fluid communication with said sample inlet port, said polynucleotide amplification region containing reagents for amplifying a polynucleotide, and lysing means intermediate the discharge section of the flow channel of said sample preparation device and said polynucleotide amplification region for lysing said cells, the discharge section of the flow channel of said sample-preparation-device being in fluid communication with the sample inlet port of said polynucleotide analysis device.
 - 55 18. The system of claim 17, which further comprises a sample flow channel in said polynucleotide analysis device interconnecting the inlet port of said device and said polynucleotide amplification region at least one of said polynucleotide amplification region and the sample flow channel connected therewith having at least one mesoscale dimension, said mesoscale dimension being a cross-sectional dimension of width or depth which is between 0.1

and 1000 µm.

19. In combination, the system of claim 11 and an appliance for use with said system, said appliance comprising a holder for said system, a test sample input conduit interfitted with the sample inlet of said sample preparation device and an impellent for moving test sample along the flow path of said sample preparation device.
20. The combination of claim 19, wherein said appliance further comprises a reservoir for said test sample.
21. In combination, the system of claim 17, and an appliance for use with said system, said appliance comprising a holder for said system, a test sample input conduit interfitted with the sample inlet of said sample preparation device, and an impellent for moving said test sample along the flow passage of said sample preparation device, a carrier fluid input conduit interfitted with said inlet section of the flow channel of said sample preparation device and an impellent for moving the said carrier fluid along said flow channel.
22. The combination of claim 21, wherein said appliance further comprises a reservoir for said carrier fluid.
23. The combination of claim 21, wherein said appliance further comprises a detector for detecting a parameter of said test sample in said analyte detection device or said polynucleotide analysis device.
24. A system for performing an analysis of preselected polynucleotide derived from cells, said analysis comprising polynucleotide amplification, said system comprising a sample preparation device according to claim 1 and a device for carrying out polynucleotide amplification which comprises:
 - a solid substrate fabricated to define:
 - a sample inlet port in fluid communication with the sample outlet of the device of claim 1 ; and a flow system comprising:
 - a polynucleotide amplification region in fluid communication with said inlet port, said polynucleotide amplification region containing reagents for amplifying a polynucleotide, and a sample flow channel interconnecting the inlet port and the polynucleotide amplification region, at least one of said sample flow channel and said polynucleotide amplification region having at least one mesoscale dimension, said mesoscale dimension being a cross-sectional dimension of width or depth which is between 0.1 and 1000 µm and lysing means in said flow channel upstream of said polynucleotide amplification region for lysing said cells, the discharge section of the flow channel of said sample preparation device being in fluid communication with the sample inlet port of said polynucleotide amplification device.

Patentansprüche

1. Vorrichtung zur Vorbereitung einer partikulären Bestandteile enthaltenden Testprobe für die Analyse, wobei die Vorrichtung einen Probenströmungsdurchgang mit einem Probeneinlaß und einem Probenauslaß, die in fluidischer Verbindung stehen, sowie einem zwischen dem Einlaß und dem Auslaß angeordneten Trennelement, wobei das Trennelement einen stromaufwärts weisenden Teil, durch den eine Trennzone in dem Strömungsdurchgang definiert ist, in der die partikulären Bestandteile gesammelt werden, sowie einen mit der Trennzone in fluidischer Verbindung stehenden Strömungskanal zur Ableitung gesammelter partikulärer Bestandteile aus der Trennzone aufweist, wobei der Kanal einen Einlaßabschnitt zum Zuführen einer Trägerflüssigkeit in die Trennzone sowie einen Ableitungsabschnitt zum Abführen der Trägerflüssigkeit von über den stromaufwärts weisenden Teil des Trennelements und aus der Trennzone heraus aufweist, wobei von dem Strömungsdurchgang und den Strömungskanalabschnitten wenigstens einer mindestens eine Abmessung im Mesomaßstab aufweist, bei der es sich um eine Querschnittsabmessung von entweder Breite oder Tiefe handelt, die zwischen 0,1 µm und 1000 µm liegt, umfaßt; dadurch gekennzeichnet, daß die Vorrichtung keine Ventile enthält; und dadurch gekennzeichnet, daß der Einlaßabschnitt des Strömungskanals so angeordnet ist, daß die Trägerflüssigkeit in die Trennzone über den stromaufwärts weisenden Teil des Trennelements geführt wird.
2. Vorrichtung nach Anspruch 1, wobei der Strömungsdurchgang mindestens eine Abmessung im Mesomaßstab aufweist und das Trennelement einen Bereich mit eingeschränkter Strömung in dem Strömungsdurchgang umfaßt, wobei der Bereich mit eingeschränkter Strömung von wenigstens einem Durchgangsweg mit mindestens einer

Abmessung im Mesomaßstab, die kleiner ist als die geringste Abmessung im Mesomaßstab des Strömungsdurchgangs und hinreichend klein ist, um die partikulären Bestandteile von der Testprobe zu trennen, wobei es sich bei den beiden Abmessungen im Mesomaßstab um eine Querschnittsabmessung von entweder Breite oder Tiefe handelt, die zwischen 0,1 µm und 1000 µm liegt, gebildet wird.

- 5 3. Vorrichtung nach Anspruch 2, wobei der wenigstens eine Durchgangsweg wenigstens eine darin liegende Biegung aufweist, so daß wenigstens ein Teil des Durchgangswegs im wesentlichen senkrecht zum Strömungskanal steht.
- 10 4. Vorrichtung nach Anspruch 1, wobei der Strömungsdurchgang und der Strömungskanal in einer Oberfläche eines festen Substrats gebildet werden und von einer an der Oberfläche haftenden Abdeckung umschlossen sind.
- 15 5. Vorrichtung nach Anspruch 4, wobei das Trennelement in Form wenigstens eines im wesentlichen aufrechten Vorsprungs des Substrats vorliegt, das in dem Strömungsdurchgang angeordnet ist und der den Fluß der Testprobe den Strömungsdurchgang entlang einschränkt.
- 16 6. Vorrichtung nach Anspruch 4, wobei die Abdeckung durchsichtig ist.
- 20 7. Vorrichtung nach Anspruch 1 in Kombination mit einem Gerät zur Verwendung mit der Vorrichtung, wobei das Gerät einen Halter für die Vorrichtung, eine mit dem Probeneinlaß der Vorrichtung zusammengepasste Leitung zum Einbringen der Testprobe sowie ein Antriebselement zum Bewegen der Testprobe den Strömungsdurchgang entlang umfaßt.
- 25 8. Kombination nach Anspruch 7, wobei das Gerät weiterhin einen Vorratsbehälter für die Testprobe umfaßt.
- 26 9. Kombination nach Anspruch 7, wobei das Gerät weiterhin eine mit dem Einlaßabschnitt des Strömungskanals zusammengepasste Leitung zum Einbringen der Trägerflüssigkeit sowie ein Antriebselement zum Bewegen der Trägerflüssigkeit den Strömungskanal entlang umfaßt.
- 30 10. Kombination nach Anspruch 7, wobei das Gerät weiterhin einen Vorratsbehälter für die Trägerflüssigkeit umfaßt.
- 31 11. System zur Bestimmung eines Analyten in einer Flüssigkeitsprobe, wobei das System eine Vorrichtung nach Anspruch 1 sowie eine Analytnachweisvorrichtung umfaßt, die
ein zur Definition
einer Probeneinlaßöffnung in fluidischer Verbindung mit dem Probenauslaß der Vorrichtung nach Anspruch 1 und
eines Strömungssystems, das
einen Analytnachweisbereich in fluidischer Verbindung mit der Probeneinlaßöffnung, wobei der Bereich ein Reagenz, das mit dem Analyten unter Erhalt eines nachweisbaren Produkts, durch das der Analyt festgelegt ist, wechselt, enthält, und einen Detektor zum Nachweis des Produkts umfaßt, hergestelltes festes Substrat umfaßt.
- 35 12. System nach Anspruch 11, in dem weiterhin die Analytnachweisvorrichtung einen die Einlaßöffnung und den Analytnachweisbereich verbindenden Probenströmungskanal enthält, wobei entweder der Analytnachweisbereich oder der Probenströmungskanal oder beide mindestens eine Abmessung im Mesomaßstab aufweisen, bei der es sich um eine Querschnittsabmessung von entweder Breite oder Tiefe handelt, die zwischen 0,1 µm und 1000 µm liegt.
- 40 13. System nach Anspruch 12, wobei es sich bei dem Reagenz um eine spezifisch an den Analyten bindende Bindungssubstanz handelt.
- 45 14. System nach Anspruch 13, wobei es sich bei dem Analyten um ein Antigen und bei der Bindungssubstanz um einen Antikörper handelt.
- 50 15. System nach Anspruch 13, wobei es sich bei dem Analyten um einen Liganden und bei der Bindungssubstanz um einen Rezeptor handelt.
- 55 16. System nach Anspruch 13, wobei es sich bei dem Analyten um ein Nukleinsäuremolekül einer vorgegebenen Sequenz und bei der Bindungssubstanz um ein Nukleinsäuremolekül mit einer zur Sequenz des Analyten komplementären oder homologen Sequenz handelt.

17. System nach Anspruch 11, das weiterhin eine Vorrichtung zur Durchführung der Analyse eines aus Zellen stammenden vorgewählten Polynukleotids umfaßt, wobei die Analyse eine Polynukleotidamplifikationsreaktion umfaßt und die Polynukleotidanalysevorrichtung ein zur Definition einer Probeneinlaßöffnung in fluidischer Verbindung mit dem Probenauslaß der Vorrichtung nach Anspruch 1 und eines Strömungssystems, das einen Polynukleotidamplifikationsbereich in fluidischer Verbindung mit der Probeneinlaßöffnung, wobei der Polynukleotidamplifikationsbereich Reagentien zur Amplifikation eines Polynukleotids enthält, und zwischen dem Ableitungsabschnitt des Strömungskanals der Probenvorbereitungsvorrichtung und dem Polynukleotidamplifikationsbereich befindliche Lysemittel zur Lyse der Zellen umfaßt, wobei der Ableitungsabschnitt des Strömungskanals der Probenvorbereitungsvorrichtung in fluidischer Verbindung mit der Probeneinlaßöffnung der Polynukleotidanalysevorrichtung steht, hergestelltes festes Substrat umfaßt.
18. System nach Anspruch 17, in dem weiterhin die Polynukleotidanalysevorrichtung einen die Einlaßöffnung der Vorrichtung und den Polynukleotidamplifikationsbereich verbindenden Probenströmungskanal enthält, wobei entweder der Polynukleotidamplifikationsbereich oder der damit verbundene Probenströmungskanal oder beide mindestens eine Abmessung im Mesomaßstab aufweisen, bei der es sich um eine Querschnittsabmessung von entweder Breite oder Tiefe handelt, die zwischen 0,1 µm und 1000 µm liegt.
19. System nach Anspruch 11 in Kombination mit einem Gerät zur Verwendung mit dem System, wobei das Gerät einen Halter für die Vorrichtung, eine mit dem Probeneinlaß der Probenvorbereitungsvorrichtung zusammengepaßte Leitung zum Einbringen der Testprobe sowie ein Antriebselement zum Bewegen der Testprobe den Strömungsweg der Probenvorbereitungsvorrichtung entlang umfaßt.
20. Kombination nach Anspruch 19, wobei das Gerät weiterhin einen Vorratsbehälter für die Testprobe umfaßt.
21. System nach Anspruch 17 in Kombination mit einem Gerät zur Verwendung mit dem System, wobei das Gerät einen Halter für das System, eine mit dem Probeneinlaß der Probenvorbereitungsvorrichtung zusammengepaßte Leitung zum Einbringen der Testprobe und ein Antriebselement zum Bewegen der Testprobe den Strömungsdurchgang der Probenvorbereitungsvorrichtung entlang, eine mit dem Einlaßabschnitt des Strömungskanals der Probenvorbereitungsvorrichtung zusammengepaßte Leitung zum Einbringen der Trägerflüssigkeit sowie ein Antriebselement zum Bewegen der Trägerflüssigkeit den Strömungskanal entlang umfaßt.
22. Kombination nach Anspruch 21, wobei das Gerät weiterhin einen Vorratsbehälter für die Trägerflüssigkeit umfaßt.
23. Kombination nach Anspruch 21, wobei das Gerät weiterhin einen Detektor zum Nachweis eines Parameters der Testprobe in der Analytnachweisvorrichtung oder der Polynukleotidanalysevorrichtung umfaßt.
24. System zur Durchführung einer Analyse eines aus Zellen stammenden vorgewählten Polynukleotids, wobei die Analyse eine Polynukleotidamplifikationsreaktion umfaßt, wobei das System eine Probenvorbereitungsvorrichtung nach Anspruch 1 sowie eine Vorrichtung zur Durchführung einer Polynukleotidamplifikation umfaßt, die ein zur Definition einer Probeneinlaßöffnung in fluidischer Verbindung mit dem Probenauslaß der Vorrichtung nach Anspruch 1 und eines Strömungssystems, das einen Polynukleotidamplifikationsbereich in fluidischer Verbindung mit der Einlaßöffnung, wobei der Polynukleotidamplifikationsbereich Reagentien zur Amplifikation eines Polynukleotids enthält, und einen die Einlaßöffnung und den Polynukleotidamplifikationsbereich verbindenden Probenströmungskanal enthält, wobei entweder der Probenvorbereitungskanal oder der Polynukleotidamplifikationsbereich oder beide mindestens eine Abmessung im Mesomaßstab aufweisen, bei der es sich um eine Querschnittsabmessung von entweder Breite oder Tiefe handelt, die zwischen 0,1 µm und 1000 µm liegt, sowie in dem Strömungskanal stromaufwärts von dem Polynukleotidamplifikationsbereich befindliche Lysemittel zur Lyse der Zellen umfaßt, wobei der Ableitungsabschnitt des Strömungskanals der Probenvorbereitungsvorrichtung in fluidischer Verbindung mit der Probeneinlaßöffnung der Polynukleotidamplifikationsvorrichtung steht, hergestelltes festes Substrat umfaßt.

Revendications

1. Dispositif pour la préparation d'un échantillon pour essai, comprenant des composants particulaires, pour l'analyse, ledit dispositif comprenant un passage pour débit d'échantillon ayant une entrée d'échantillon et une sortie en communication fluide et un séparateur disposé entre ladite entrée et ladite sortie, ledit séparateur ayant une partie ascendante faisant face définissant une zone de séparation dans ledit passage pour débit dans lequel lesdits composants particulaires sont récoltés et un canal en communication fluide avec ladite zone de séparation pour pouvoir décharger les composants particulaires récoltés de ladite zone de séparation, ledit canal ayant une section d'entrée pour diriger un fluide de transport dans ladite zone de séparation, de dessus la partie ascendante faisant face dudit séparateur et une section de décharge pour diriger ledit fluide de transport en dehors de ladite zone de séparation, au moins une de ladite section de passage pour débit et de ladite section de canal pour débit ayant au moins une dimension de mésocéphale, ladite dimension de mésocéphale étant une dimension transversale de largeur ou de profondeur qui est entre 0,1 et 1000 µm, **caractérisé en ce que** le dispositif n'inclut pas de valves et **caractérisé en ce que** ladite section d'entrée dudit canal pour débit est disposée pour diriger ledit fluide de transport dans ladite zone de séparation sur la partie ascendante faisant face dudit séparateur.
2. Dispositif selon la revendication 1, dans lequel ledit passage pour débit a au moins une dimension de mésocéphale et ledit séparateur comprend une région de débit restreint dans ledit passage pour débit, ladite région de débit restreint étant formée par au moins une voie de passage ayant au moins une dimension de mésocéphale, qui est plus petite que l'au moins une dimension de mésocéphale dudit passage pour débit et qui est suffisamment petite pour séparer lesdits composants particulaires dudit échantillon pour essai, les deux dimensions de mésocéphale étant une dimension transversale de largeur ou de profondeur qui est entre 0,1 et 1000 µm.
3. Dispositif selon la revendication 2, dans lequel ladite au moins une voie de passage a au moins un coude de sorte qu'au moins une partie de ladite voie de passage est généralement perpendiculaire audit passage pour débit.
4. Dispositif selon la revendication 1, dans lequel ledit passage pour débit et ledit canal pour débit sont formés dans une surface d'un substrat solide et enfermés par un couvercle qui adhère à ladite surface.
5. Dispositif selon la revendication 4, dans lequel ledit séparateur est sous la forme d'au moins une projection généralement droite dudit substrat qui est disposé dans ledit passage pour débit et qui restreint le débit de l'échantillon pour essai le long dudit passage pour débit.
6. Dispositif selon la revendication 4, dans lequel ledit couvercle est transparent.
7. Combinaison du dispositif selon la revendication 1 et d'un appareil pour l'utilisation avec ledit dispositif, ledit appareil comprenant un support pour ledit dispositif, un conduit d'entrée d'échantillon pour essai adapté à l'entrée de l'échantillon dudit dispositif et un dispositif pour mettre en mouvement l'échantillon pour essai le long dudit passage pour débit.
8. Combinaison selon la revendication 7, dans laquelle ledit appareil comprend en outre un réservoir pour ledit échantillon pour essai.
9. Combinaison selon la revendication 7, dans laquelle ledit appareil comprend en outre un conduit d'entrée du fluide de transport adapté à ladite section d'entrée dudit canal et un dispositif pour mettre en mouvement le fluide de transport le long dudit canal.
10. Combinaison selon la revendication 7, dans laquelle ledit appareil comprend en outre un réservoir pour ledit fluide de transport.
11. Système pour la détermination d'un analyte dans un échantillon fluide, ledit système comprenant un dispositif selon la revendication 1 et un dispositif de détection d'analyte dans lequel ledit dispositif de détection d'analyte comprend :
- un substrat solide fabriqué pour définir :

un orifice d'entrée d'échantillon en communication fluide avec la sortie de l'échantillon du dispositif selon la revendication 1 ; et un système de débit comprenant : une région de détection d'analyte en commun-

cation fluide avec ledit orifice d'entrée de l'échantillon, ladite région contenant un réactif qui interagit avec ledit analyte pour donner un produit détectable qui est déterminant dudit analyte et un détecteur pour détecter ledit produit.

- 5 12. Système selon la revendication 11, qui comprend en outre, dans ledit dispositif de détection d'analyte, un canal d'échantillon interconnectant ledit orifice d'entrée et ladite région de détection d'analyte, au moins un de la dite région de détection de l'analyte et dudit canal d'échantillon ayant au moins une dimension de mésoéchelle, ladite dimension de mésoéchelle étant une dimension transversale de largeur ou de profondeur qui est entre 0,1 et 1000 µm.
- 10 13. Système selon la revendication 12, dans lequel ledit réactif est une substance de liaison qui se lie spécifiquement audit analyte.
- 15 14. Système selon la revendication 13, dans lequel ledit analyte est un antigène et ladite substance de liaison est un anticorps.
- 20 15. Système selon la revendication 13, dans lequel ledit analyte est un ligand et ladite substance de liaison est un récepteur.
- 25 16. Système selon la revendication 13, dans lequel ledit analyte est une molécule d'acide nucléique de séquence prédéterminée et ladite substance de liaison est une molécule d'acide nucléique ayant une séquence complémentaire ou homologue à la séquence dudit analyte.
- 30 17. Système selon la revendication 11 comprenant en outre un dispositif pour effectuer l'analyse de polynucléotide présélectionné dérivé de cellules, ladite analyse comprenant une réaction d'amplification de polynucléotide, ledit dispositif d'analyse de polynucléotide comprenant :
- 35 un substrat solide fabriqué pour définir :
- 40 un orifice d'entrée d'échantillon en communication fluide avec la sortie d'échantillon du dispositif selon la revendication 1 ; et un système de débit comprenant :
- 45 une région d'amplification de polynucléotide en communication fluide avec ledit orifice d'entrée d'échantillon, ladite région d'amplification de polynucléotide contenant des réactifs pour amplifier un polynucléotide, et des moyens de lyse intermédiaires à la section de décharge du canal pour débit dudit dispositif de préparation d'échantillon et ladite région d'amplification de polynucléotide pour lyser lesdites cellules, la section de décharge du canal dudit dispositif de préparation de l'échantillon étant en communication fluide avec l'orifice d'entrée de l'échantillon dudit dispositif d'analyse de polynucléotide.
- 50 18. Système selon la revendication 17, qui comprend en outre un canal pour débit d'échantillon dans ledit dispositif d'analyse de polynucléotide interconnectant l'orifice d'entrée dudit dispositif et ladite région d'amplification de polynucléotide au moins une de ladite région d'amplification de polynucléotide et dudit canal d'échantillon connecté avec lui ayant au moins une dimension de mésoéchelle, ladite dimension de mésoéchelle étant une dimension transversale de largeur ou profondeur qui est entre 0,1 et 1000 µm.
- 55 19. Combinaison du système selon la revendication 11 et d'un appareil pour l'utilisation avec ledit système, ledit appareil comprenant un support pour ledit système, un conduit d'entrée d'échantillon adapté à l'entrée d'échantillon pour essai dudit dispositif de préparation d'échantillon et un dispositif pour mettre en mouvement l'échantillon pour essai le long du passage pour débit dudit dispositif de préparation d'échantillon.
- 60 20. Combinaison selon la revendication 19, dans laquelle ledit appareil comprend en outre un réservoir pour ledit échantillon pour essai.
- 65 21. Combinaison du système selon la revendication 17 et d'un appareil pour l'utilisation avec ledit système, ledit appareil comprenant un support pour ledit système, un conduit d'entrée d'échantillon pour essai adapté à l'entrée d'échantillon dudit dispositif de préparation d'échantillon et un dispositif pour mettre en mouvement ledit échantillon pour essai le long du passage pour débit dudit dispositif de préparation d'échantillon, un conduit d'entrée de fluide

de transport adapté à ladite section d'entrée du canal dudit dispositif de préparation d'échantillon et un dispositif pour mettre en mouvement ledit fluide de transport le long dudit canal.

22. Combinaison selon la revendication 21, dans laquelle ledit appareil comprend en outre un réservoir pour ledit fluide de transport.

10 23. Combinaison selon la revendication 21, dans laquelle ledit appareil comprend en outre un détecteur pour déetecter un paramètre dudit échantillon pour essai dans ledit dispositif de détection d'analyte ou dudit dispositif d'analyse de polynucléotide.

15 24. Système pour effectuer une analyse de polynucléotide présélectionné dérivé de cellules, ladite analyse comprenant l'amplification du polynucléotide, ledit système comprenant un dispositif de préparation d'échantillon selon la revendication 1 et un dispositif pour effectuer l'amplification de polynucléotide qui comprend :

un substrat solide fabriqué pour définir :

un orifice d'entrée d'échantillon en communication fluide avec la sortie d'échantillon du dispositif selon la revendication 1 ; et un système de débit comprenant :

20 une région d'amplification de polynucléotide en communication fluide avec ledit orifice d'entrée, ladite région d'amplification de polynucléotide contenant des réactifs pour amplifier un polynucléotide, et un canal d'échantillon interconnectant l'orifice d'entrée et la région d'amplification de polynucléotide, au moins un dudit canal d'échantillon et de ladite région d'amplification du polynucléotide ayant au moins une dimension de mésoéchelle, ladite dimension de mésoéchelle étant une dimension transversale de largeur ou de profondeur qui est entre 0,1 et 1000 µm et des moyens de lyse dans ledit canal pour débit en amont de ladite région d'amplification de polynucléotide pour lyser lesdites cellules, la section de décharge du canal pour débit dudit dispositif de préparation d'échantillon étant en communication fluide avec l'orifice d'entrée d'échantillon dudit dispositif d'amplification de polynucléotide.

30

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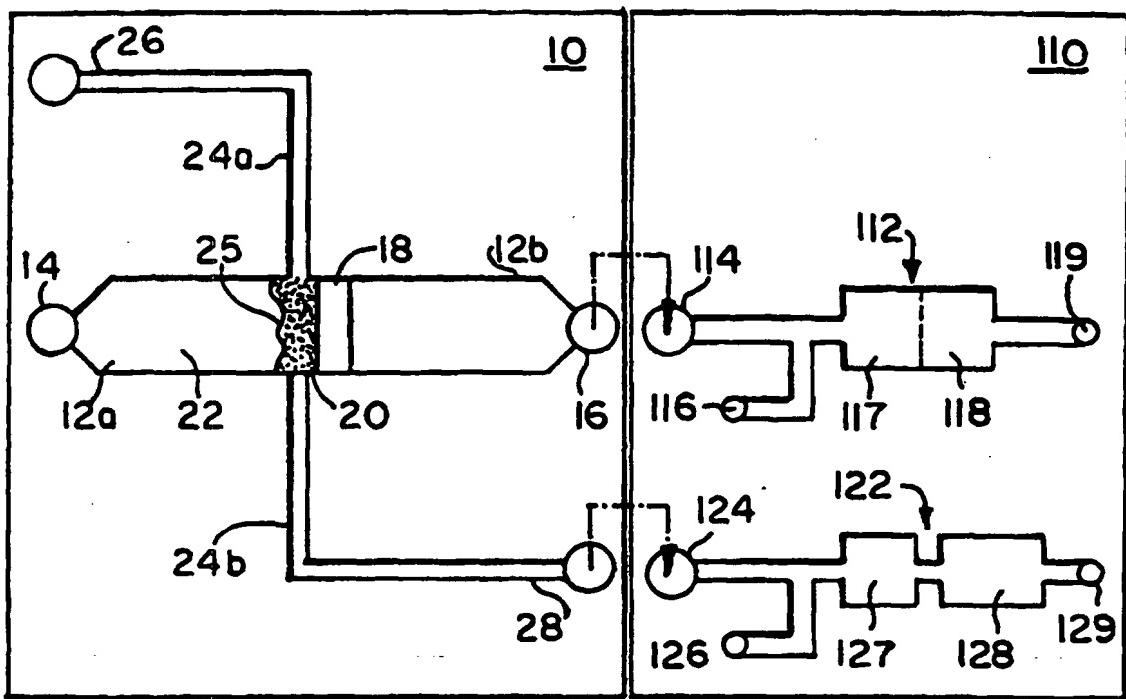
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FIG. 5



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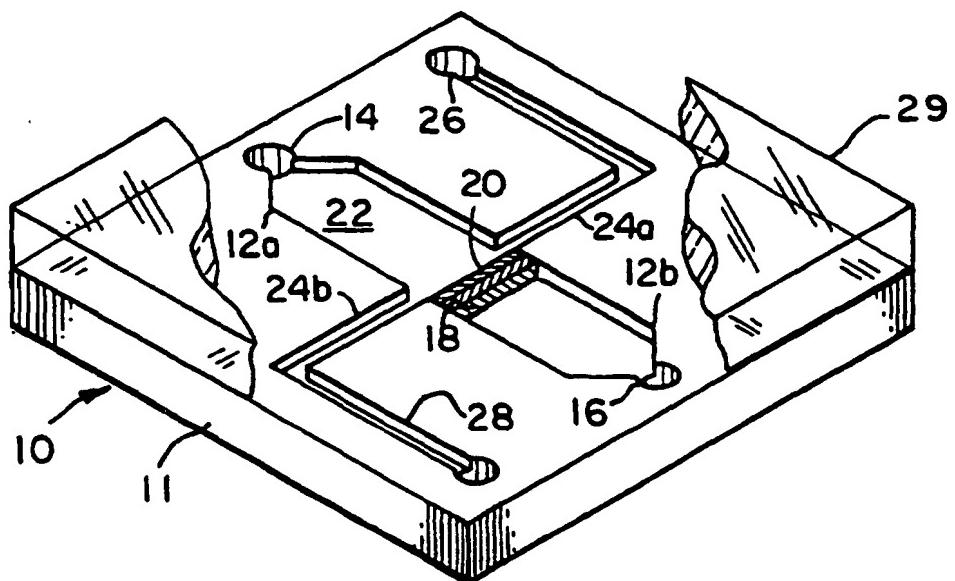


FIG. 1

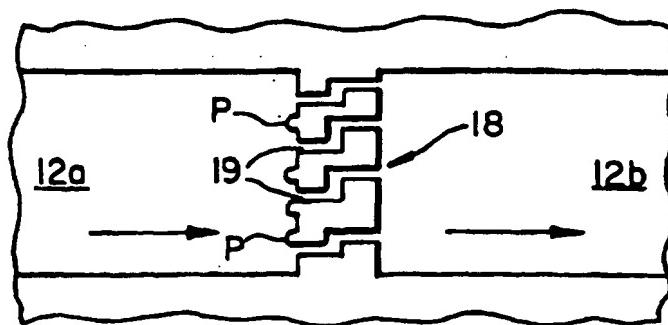


FIG. 2

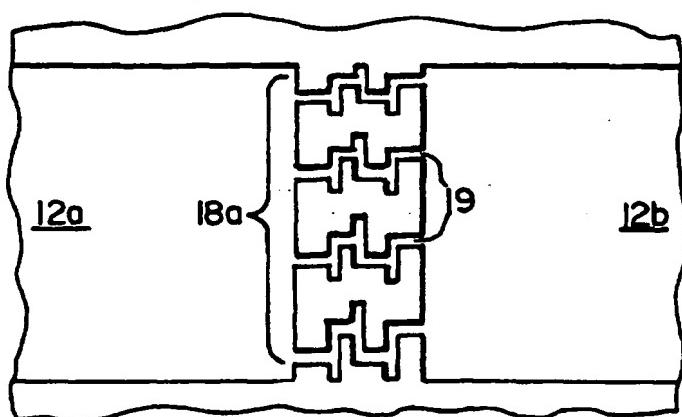


FIG. 3

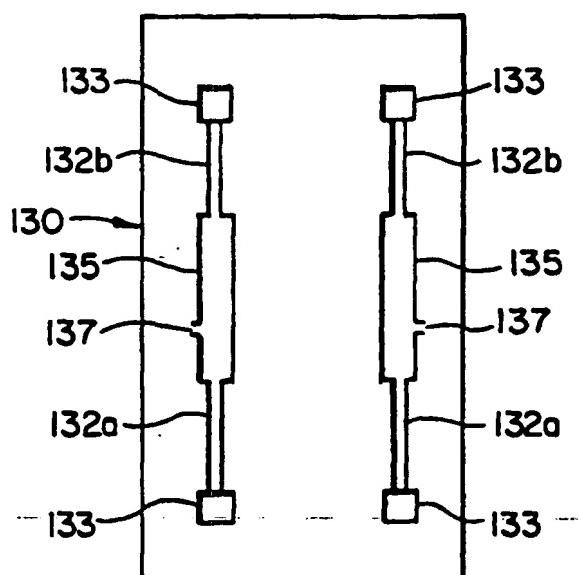


FIG. 8A

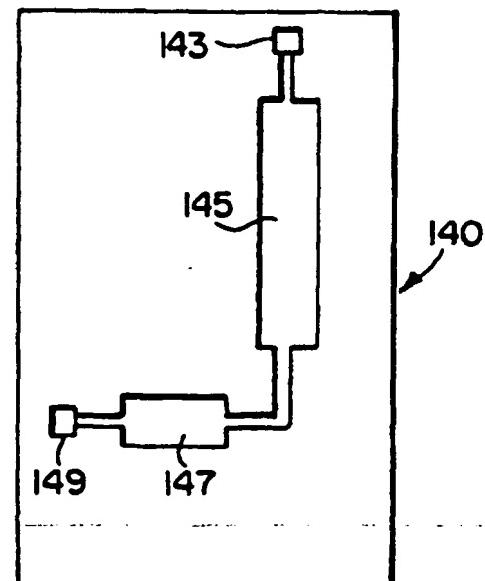


FIG. 8B

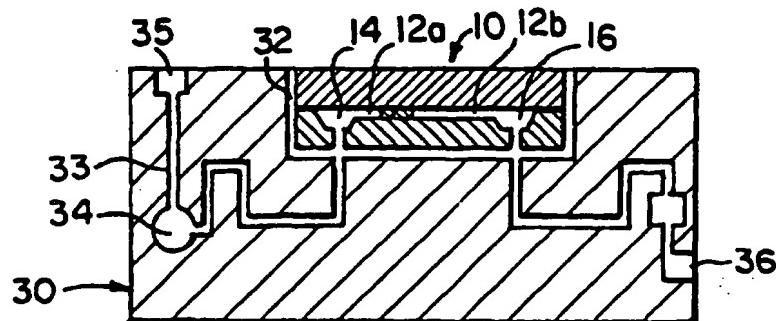


FIG. 4

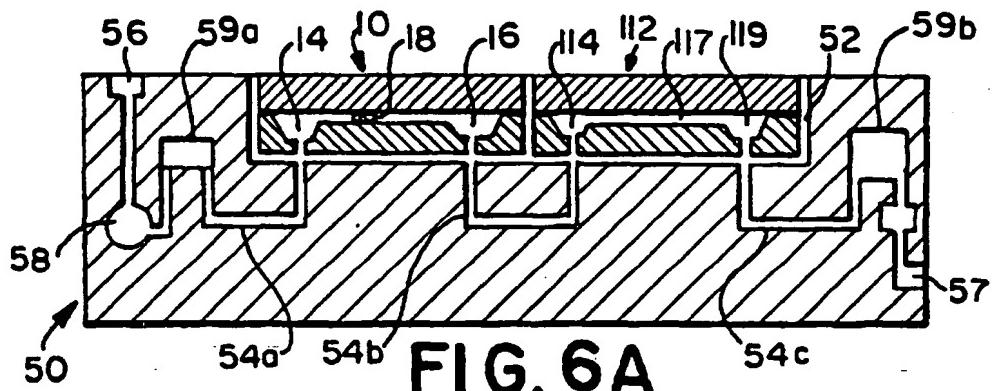


FIG. 6A

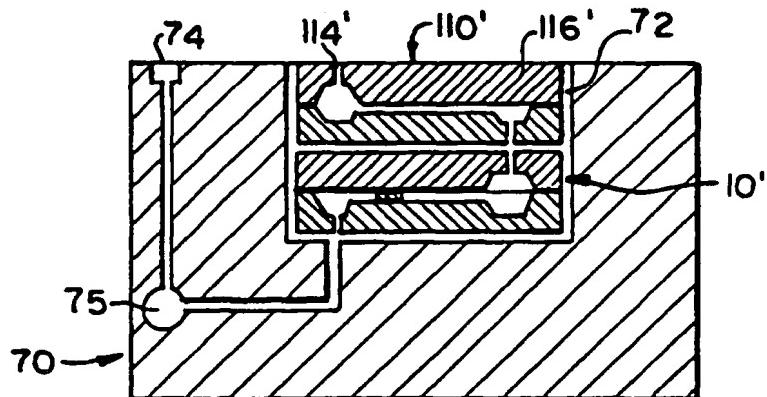


FIG. 6B

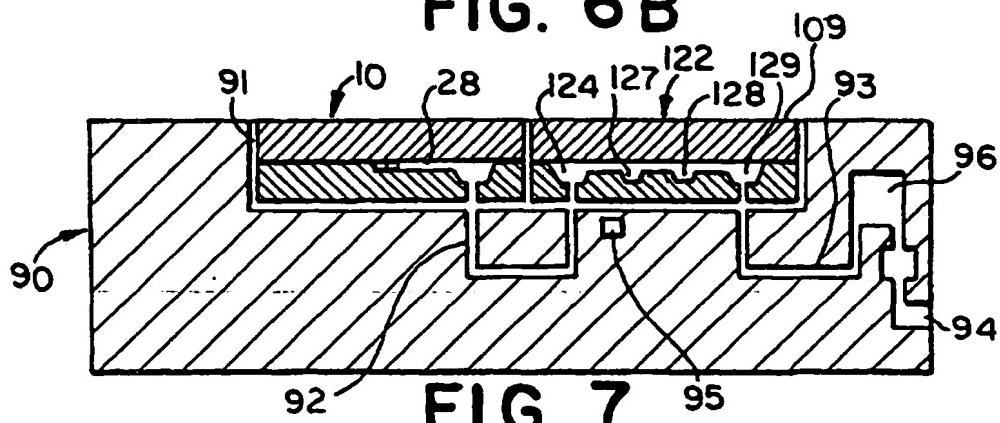


FIG. 7

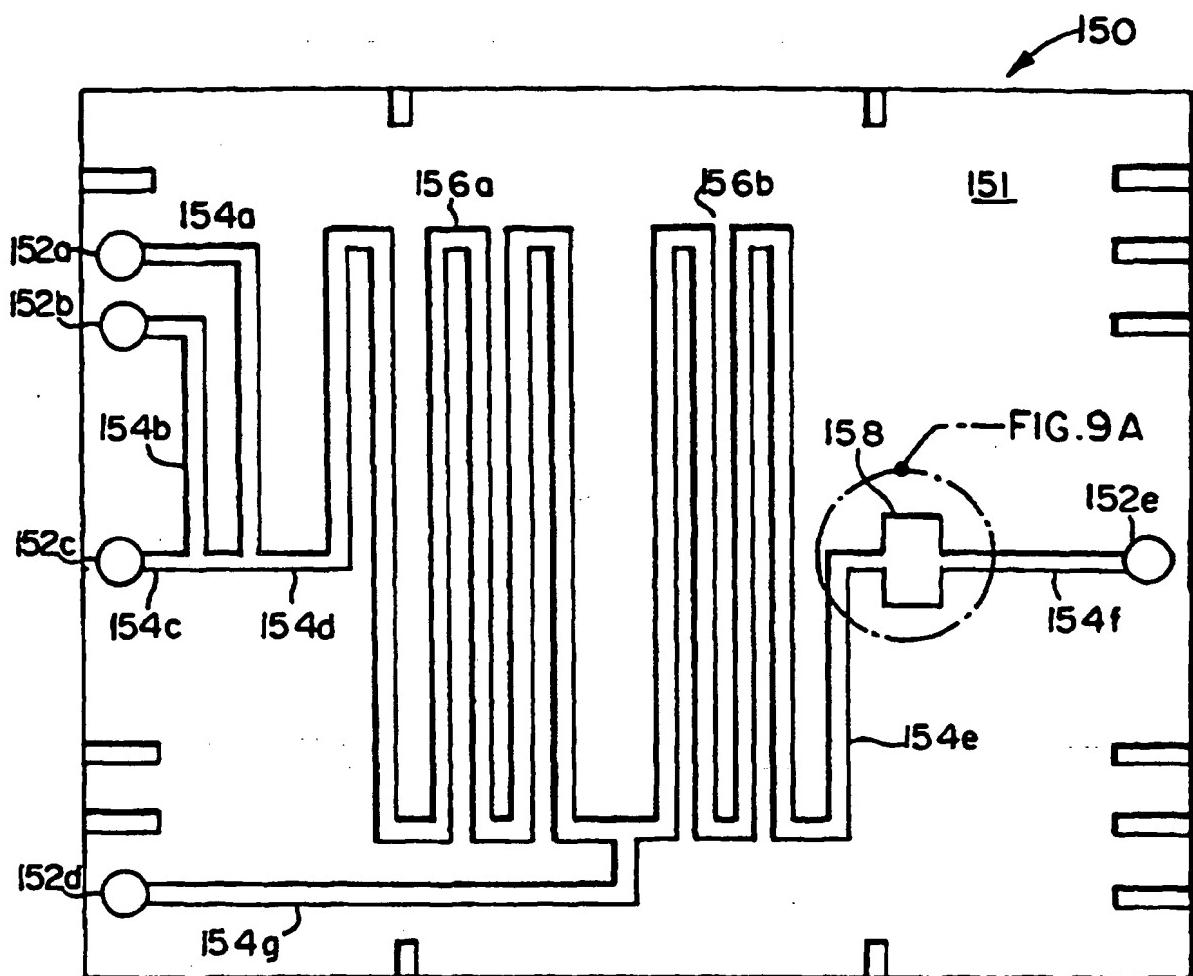


FIG. 9

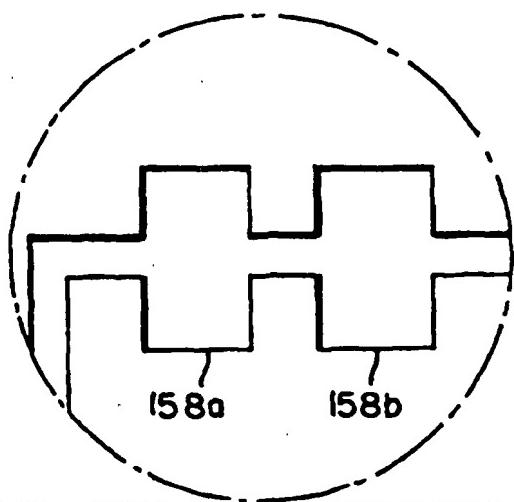


FIG. 9B

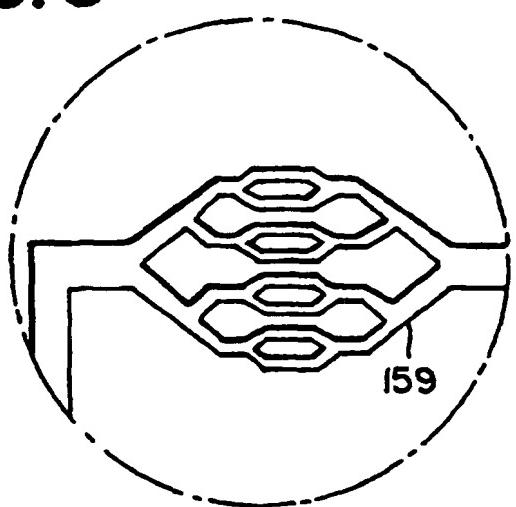


FIG. 9C

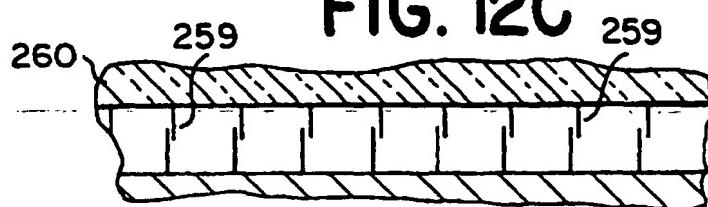
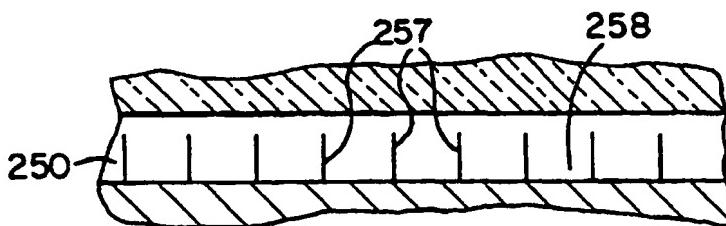
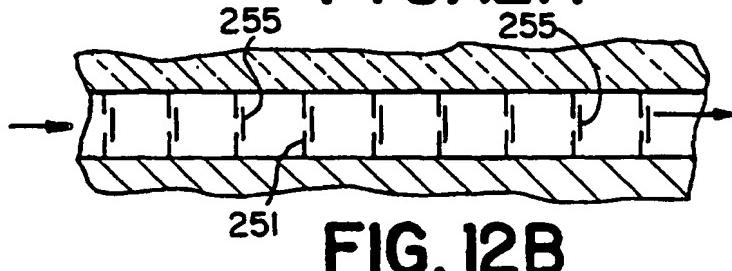
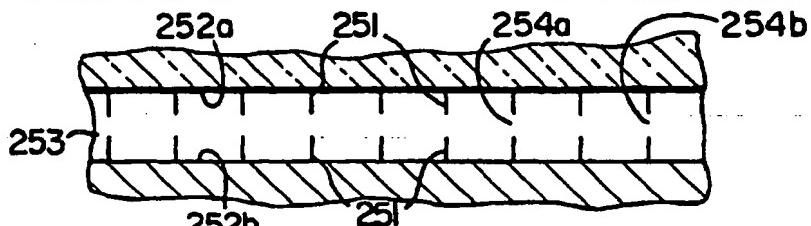
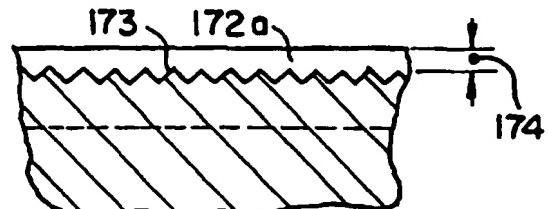
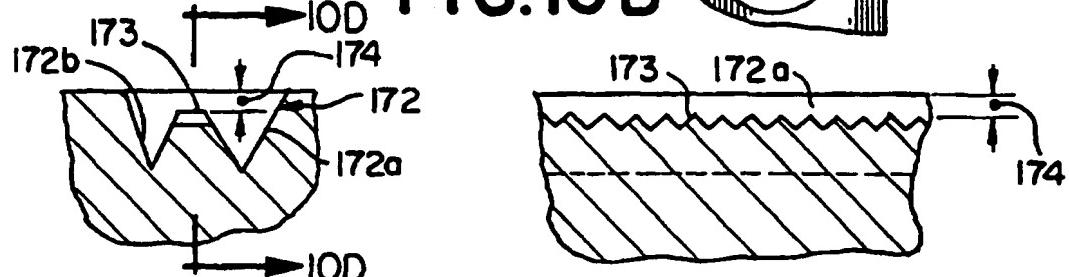
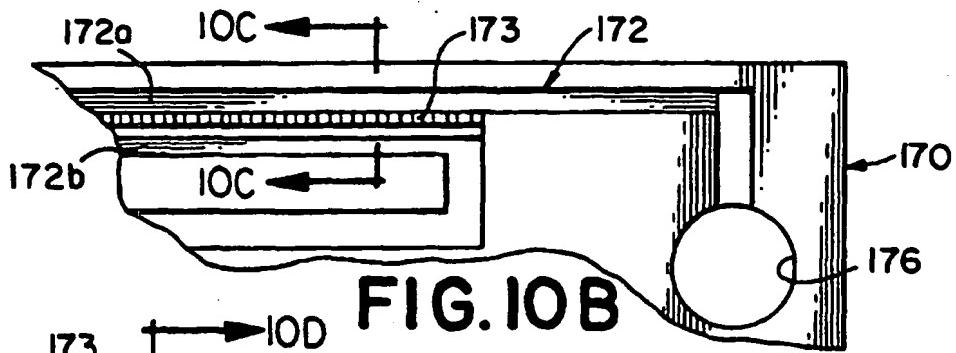


FIG. 10A

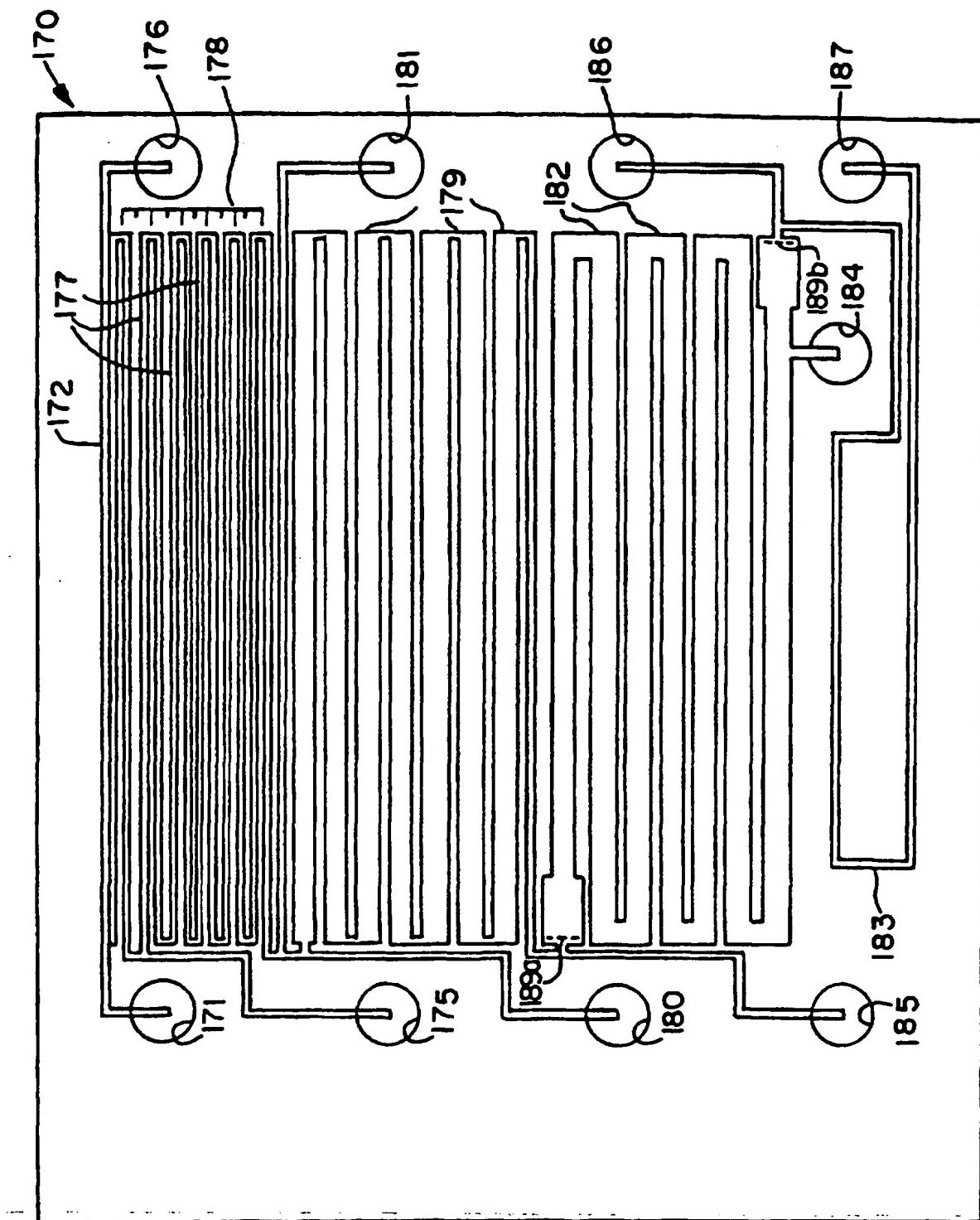


FIG. 11A

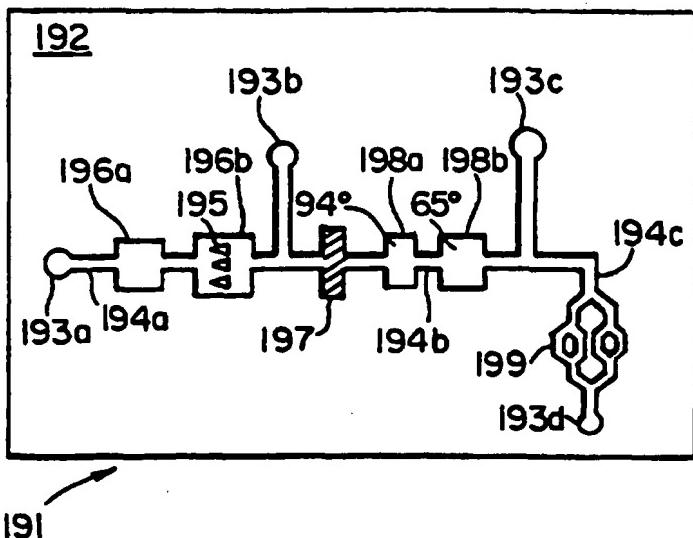
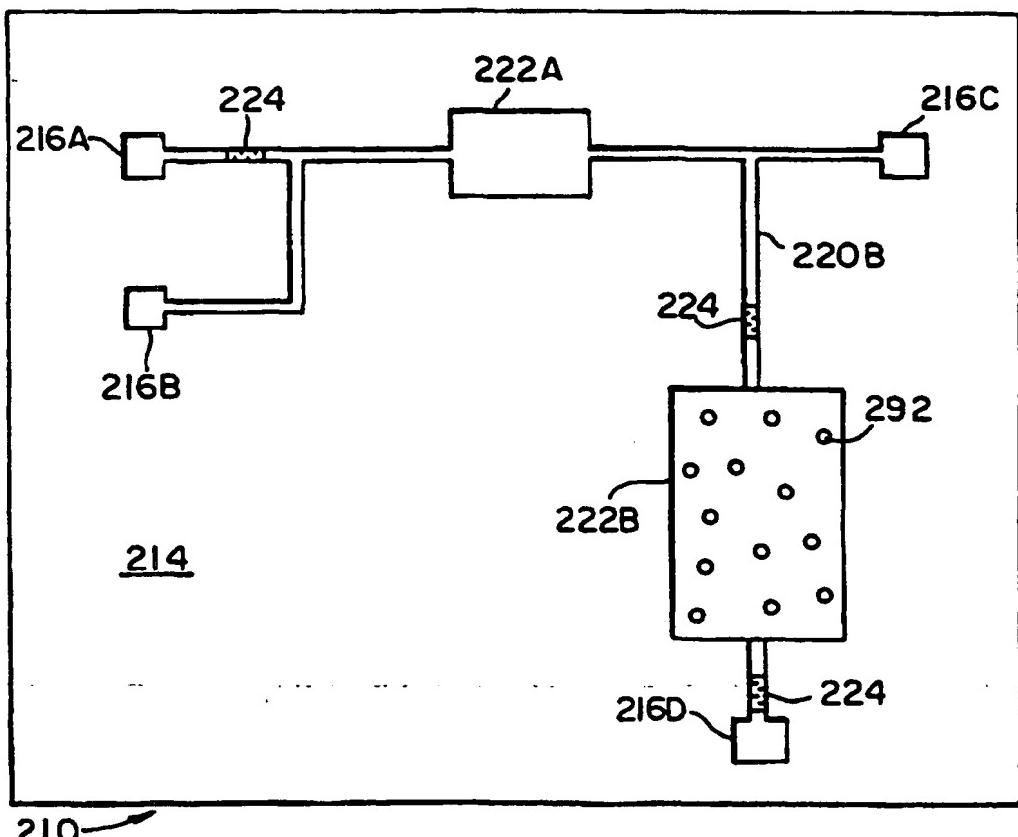


FIG. 11B



(19)



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under INID code 62.

(54) Antigen specific separation of nucleated fetal cells in non-invasive detection of fetal DNA

(57) A method of isolating fetal nucleated cells, particularly fetal nucleated erythrocytes, from a maternal blood sample, by means of an antigen present on the cell surface of the fetal erythrocytes. A method of detecting fetal DNA of interest, which is a gene or gene portion associated with a disease or condition, a chromosomal abnormality or sex-specific DNA, in a maternal blood sample. The presence or absence, as well as the quantity of fetal DNA of interest in a maternal sample can be determined. The claimed method of detection can be used prenatally or postnatally and is particularly useful because it is non-invasive and can be carried out early in pregnancy.

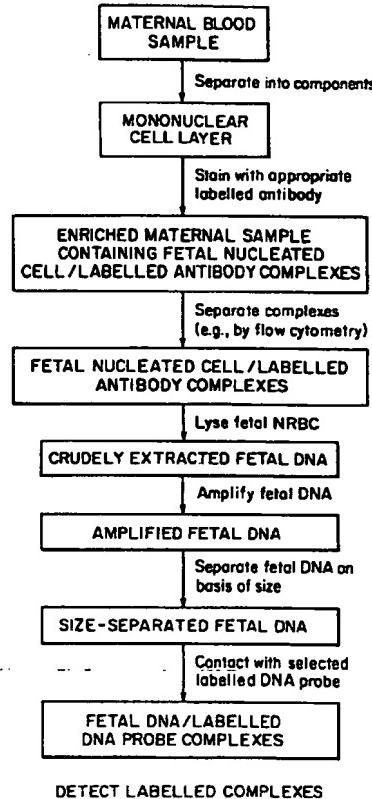


Fig. 1

EP 0 791 659 A2

DescriptionFunding

5 Work described herein was supported by the National Institutes of Health and Children's Hospital Medical Center.

Background

A variety of fetal cell types--platelets, trophoblasts, erythrocytes and leucocytes--cross the placenta and circulate
 10 transiently within maternal blood (Schroder, J., J. Med. Genet., 12:230-242 (1975); Douglas G.W. et al., Am. J. Obstet. Gynec., 78:960-973 (1959)). There have been numerous reports of efforts to separate fetal cells from maternal cells present in maternal blood, but none has been successful in isolating cells subsequently shown to contain fetal DNA. Distinguishing fetal cells from maternal cells has not been successful for several reasons, including the small number
 15 of fetal cells in a maternal blood sample and the fact that morphological differences are slight (e.g., trophoblasts are the only fetal cells which can be distinguished from maternal cells by morphology alone).

Others report screening the peripheral blood of pregnant women for cells of fetal origin. Fetal identification relied on the presence of a single cytogenetic marker, the Y chromosome. Lymphocytes with a putative "XY" karyotype were found in the maternal circulation as early as 14 weeks gestation (Walknowska, J., et al., The Lancet, 1119-1122 (1979)).

20 The availability of flow cytometry has led many to suggest that fetal cells could be obtained through the use of a flow cytometer and that such cells could be exploited for prenatal genetic diagnosis. However, although cells sorted in this manner have been said to be of fetal origin, based on analysis of cell surface antigens, morphology, or cytogenetic criteria, there has not been confirmation that the cells contain fetal DNA. A method by which fetal DNA could be obtained from maternal blood during pregnancy would be valuable, particularly if it made it possible to carry out prenatal
 25 diagnosis by a noninvasive technique.

Disclosure of the Invention

The present invention is an in vitro method of separating or isolating fetal DNA present in the blood of a pregnant
 30 woman from maternal DNA, as well as an in vitro method of detecting the presence of and/or quantitating selected fetal DNA in fetal DNA, which is useful as a noninvasive prenatal diagnostic or analytical method.

In the present method, fetal nucleated cells are isolated from a maternal blood sample by means of a detectable material which binds to the fetal nucleated cells but not to maternal cells and is then separated from the maternal sample, resulting in separation of the fetal nucleated cells from the sample. The fetal nucleated cells can be any undifferentiated hematopoietic cell and, particularly, fetal nucleated erythrocytes. In one embodiment of the present method of isolation, at least one detectably labelled monoclonal antibody specific for an antigen present on fetal nucleated cells, but not for an antigen present on maternal cells, is combined with a maternal blood sample and, once bound to fetal nucleated cells, is separated from the maternal sample. Alternatively, at least one detectably labelled monoclonal antibody specific for an antigen present on maternal cells, but not for an antigen present on fetal nucleated cells is used. In
 40 a further embodiment, the two types of monoclonal antibodies are used.

In the case in which the detectable label is a fluorescent molecule, separation is carried out by means of flow cytometry, in which fluorescently-labelled molecules are separated from unlabelled molecules. This results in separation of fetal nucleated cells, such as fetal nucleated erythrocytes, from maternal cells and, thus, of fetal DNA from maternal DNA. That this separation has occurred can be verified using known techniques, such as microscopy or detection
 45 of fetal hemoglobin.

In one embodiment of the method of the present invention by which the occurrence of a selected DNA sequence or sequences (gene(s) or gene portion(s)) in fetal DNA is determined (detected and/or quantitated), the isolated fetal nucleated cells, such as fetal nucleated erythrocytes, are treated to render DNA present in them available for amplification. Amplification of DNA from fetal nucleated cells (fetal DNA) is carried out using a known amplification technique, such as the polymerase chain reaction (PCR). Amplified fetal nucleated cell DNA is subsequently separated on the basis of size (e.g., by gel electrophoresis) and contacted with a selected labelled probe, such as labelled DNA complementary to a selected DNA sequence (e.g., complementary to an abnormal gene or gene portion, or Y-specific DNA). Detection of the labelled probe after it has hybridized to fetal DNA results in detection of the sequence of interest in the fetal DNA. Quantitation of the hybridized labelled probe results in quantitation of the fetal DNA.

55 In a second embodiment of the present method of determining the occurrence of a selected DNA sequence (or sequences), cells isolated as described above are sorted onto a solid support, such as a slide, and screened for chromosomal abnormalities using in situ hybridization. In this embodiment, a selected nucleic acid probe, such as a labelled DNA probe for chromosomal DNA associated with a congenital abnormality, is combined with the fetal DNA, under conditions appropriate for hybridization of complementary sequences to occur. Detection and/or quantitation of the labelled

probe after hybridization results in detection and/or quantitation of the fetal DNA to which the probe has hybridized.

The present method of detecting the occurrence of selected fetal DNA is useful for prenatal evaluation or diagnostic purposes, such as determination of the sex of the fetus, assessment of chromosomal abnormalities and determination of the presence of abnormal genes associated with human disease.

5 A particular advantage of the method of the present invention by which fetal nucleated erythrocytes are isolated is that such cells can be reliably separated from cells of maternal origin. In addition, because such cells are nucleated and, thus, contain a full complement of fetal genes, the present method makes available complete fetal DNA. The present method of detecting and/or quantitating a selected fetal DNA sequence is particularly valuable not only because of the advantages associated with the present method of isolating fetal cells, but also because it is a noninvasive technique which can be applied early in gestation.
10

Brief Description of the Drawings

15 Figure 1 is a schematic representation of the method of the present invention by which fetal nucleated cells are isolated from maternal cells and DNA within the fetal cells is assessed for the occurrence of a particular fetal DNA sequence.

Figure 2 is an autoradiograph of diluted male DNA amplified for 222 bp sequence. Lane 1: reagent control; lane 2: ϕ X174 molecular weight standard; lane 3: 100 ng; lane 4: 10 ng; lane 5: 1 ng; lane 6: 200 pg; lane 7: 10 pg; lane 8: 1 pg.

20 Figure 3 is a composite autoradiograph of amplified patient DNA. Lane 1: 10 ng normal male; lane 2: 10 ng normal female; lane 3: reagent control; lane 4: ϕ X174; lane 5: sorted cells from patient 1 (male fetus); lane 6: sorted cells from patient 2 (male fetus); lane 7: sorted cells from patient 3 (female fetus); lane 8: sorted cells from patient 6 (female fetus); lane 9: sorted cells from patient 7 (male fetus); lane 10: sorted cells from patient 8 (male fetus); lane 11: sorted cells from patient 9 (female fetus); lane 12: cord blood from female infant whose cells were prenatally sorted in lane 8.

25 Figure 4 is a diagram demonstrating the detection of Y chromosomal DNA sequences at various points of gestation in women bearing male pregnancies.

Figure 5 is a series of histograms (A through F) obtained when FITC-anti transferrin receptor was used to determine the presence of mononuclear cells in samples from non-pregnant females to which male cells have been added.

Figure 6 is a composite autoradiograph of amplified male DNA detected in TfR⁺ cells when 10^2 - 10^6 male cells are added to samples from non-pregnant females and in TfR⁻ cells when 10^5 - 10^6 male cells are added to samples from non-pregnant females.
30

Figure 7 is a series of histograms (A through H) obtained when anti HPCA-1 antibody was used to determine the presence of mononuclear cells in samples from non-pregnant females to which male cells have been added.

Figure 8 is a photograph illustrating a fluorescent cell due to the positive results of *in situ* hybridization of the pDP97 probe for the Y chromosome to a fetal nucleated red blood cell.
35

Detailed Description of the Invention

40 The present invention relates to an *in vitro* method of separating or isolating fetal nucleated cells present in the blood of a pregnant woman (a maternal blood sample) from the pregnant woman's cells and of separating or isolating fetal DNA from maternal DNA. It further relates to an *in vitro* method of prenatal detection and/or quantitation of selected fetal DNA in fetal DNA isolated from the maternal blood sample. The method provides a noninvasive approach to detect and/or quantitate fetal DNA, such as that associated with a disease or a condition whose assessment during gestation is desired. It also provides a noninvasive means by which the sex of a fetus can be determined.

45 The following is a description of the basis for the subject method; of the present method of isolating nucleated fetal cells present in the blood of a pregnant woman from maternal cells and, subsequently, separating fetal DNA from maternal DNA; and of the present method of prenatal determination of the occurrence (presence/absence or quantitation) of selected DNA in fetal cells.

Nucleated erythrocyte as a potential source of fetal genes

50 It has now been determined that fetal nucleated cells, present in the blood of a pregnant woman are a source of fetal genes. That is, it has been shown that fetal nucleated erythrocytes (also referred to as fetal NRBC) can be isolated or separated from maternal blood and that DNA present in the isolated fetal cells can be used to assess fetal characteristics (e.g., sex, presence or absence of chromosomal abnormalities).

Fetal nucleated erythrocytes were selected for sorting based on the following rationale:

1. In any given fetomaternal hemorrhage, no matter how small, the ratio of fetal erythrocytes to fetal lymphocytes should remain the same as in whole fetal blood; thus, there would be 1,000 times as many red cells as white cells

available for analysis.

2. Normal pregnant females do not usually have circulating NRBC; therefore, an isolated NRBC would *a priori* have a greater chance of being fetal in origin.

5 3. The majority of pregnancies are blood group compatible, which means that the "transfused" NRBC would probably be tolerated by the mother and remain in her circulation.

10 4. Because they are nucleated, the NRBC contain a full complement of fetal genes.

Advances in molecular biology applied to fetal cell sorting

Recent advances in molecular biology have had an enormous impact on the feasibility of fetal cell identification. For example, fluorescent *in situ* hybridization can be used for this purpose.

15 The development of the polymerase chain reaction (PCR) (Mullis, K., et al., *Cold Spring Harb. Symp. Quant. Biol.*, **51**:263-272 (1986)), with its capacity for DNA analysis from a single cell (Li, H., et al., *Nature*, **355**:414-417 (1988); Handyside, A.H., et al., *Lancet* **1**:347-349 (1989)), has eliminated the technical problems associated with the small number of fetal cells in maternal blood. It makes DNA diagnosis from a single cell possible.

20 As described below, fetal nucleated erythroblasts have been shown to be present in blood obtained from pregnant women, thus making maternal blood a useful/reliable potential source of fetal DNA; fetal nucleated cells have been distinguished from maternal cells on the basis of surface antigenic characteristics, thus making it possible to separate the two cell types from one another; and fetal DNA present in the separated fetal nucleated cells has been analyzed and characterized.

Detection of fetal gene sequences in maternal blood

25 One of the first steps in developing the present method of isolating fetal nucleated cells from the maternal blood supply was identification of monoclonal antibodies that permit identification and separation of fetal cells from maternal cells present in blood obtained from a pregnant woman. This has been done, as described in detail in the Examples.

30 As a result, it has been determined that monoclonal antibodies which recognize maternal leucocytes and monoclonal antibodies which recognize fetal cell surface antigens are useful in separating maternal and fetal cells. The following is a brief description of monoclonal antibodies which have been shown to be useful in separating fetal nucleated cells from maternal cells present in a maternal blood sample. However, other monoclonal antibodies which distinguish between fetal and maternal cells on the basis of surface antigenic differences, can also be used in the present method. The 35 present method requires the use of at least one type of antibody which is specific for (or recognizes) a surface antigen present on fetal nucleated cells, for a surface antigen present on maternal cells, but not specific for both. That is, the present method can be carried out using one or more antibody which distinguishes fetal nucleated cells from maternal cells. The present method can be carried out using whole blood or blood treated or processed to enrich for (increase the concentration of) fetal nucleated cells.

40 Described below is the selection and successful use of monoclonal antibodies which distinguish fetal nucleated erythrocytes from maternal cells. It is to be understood, however, that in a similar manner, monoclonal antibodies which make it possible to select for another fetal nucleated cell type (or types) can be identified and used in the present method to separate fetal nucleated cells from maternal cells (and, thus, fetal DNA sources from maternal DNA).

45 Initial efforts focused on the elimination of contaminating maternal leucocytes in the mononuclear cell layer and identification of monoclonal antibodies effective in carrying out this separation, which results in production of a maternal sample enriched in fetal nucleated cells.

50 HLe-1 (Becton-Dickinson Monoclonal center, Mountain View, CA, catalog #7463) is a monoclonal antibody available as a direct fluorescein isothiocyanate (FITC) conjugate. It recognizes an antigen present on mature human leucocytes and on very immature erythrocyte precursors, but not on mature nucleated erythrocytes (Loken, M.E., et al., *Blood*, **69**:255-263 (1987)). Thus, maternal leucocytes are recognized and bound, but fetal nucleated erythrocytes are not, making separation of the two possible. As described in detail in Example 1, this labelled antibody was used to eliminate maternal leucocytes in the mononuclear cell layer.

55 As is also described (Example 1), a combination of monoclonal antibodies has been used for the same purpose (i.e., elimination of maternal cells from the blood sample). As described, anti-monocyte antibody (M3) and anti-lymphocytes antibody (L4) have been used to remove maternal cells from the mononuclear cell layer resulting from density gradient centrifugation.

Monoclonal antibodies which recognize fetal nucleated cells but do not recognize maternal cells were also identified. As described in detail in Example 1, a monoclonal antibody which recognizes the transferrin receptor was identified. Erythroblasts have been shown to express the transferrin receptor (Loken, M.R., et al., *Blood*, **69**:255-263 (1987))

antigen on their cell surfaces from the BFU-E stage until nuclear extrusion (Loken, M.R. et al., Blood, **69**:255-263 (1987)). The transferrin receptor is also present on activated lymphocytes (Trowbridge, I.S. and M.B. Ormrod, Proc. Natl. Acad. Sci. USA, **78**:3039-3043 (1981)), certain tumor cells (Greaves, M. et al., Int. J. Immunopharmac., **3**:283-300 (1981)), and trophoblast cells (Galbraith, G.M.P. et al., Blood, **55**:240-242 (1980)). Thus, such an antibody is specific for or recognizes (binds to) fetal nucleated cells, but not maternal leucocytes. As described in Example 1, commercially available fluorescein-conjugated monoclonal antibodies against the transferrin receptor (TfR) were used to separate fetal nucleated erythrocytes from maternal cells. Although the antibody is not specific for fetal nucleated erythrocytes, it facilitated their enrichment in the flow-sorted samples. Other monoclonal antibodies which are able to distinguish between fetal nucleated cells and maternal cells present in a blood sample can also be used. Such antibodies include commercially available monoclonal antibodies and those which can be produced using known techniques.

Separation of fetal nucleated cells from a maternal blood sample using antibodies described above can be carried out with samples of whole blood or a fraction of whole blood (i.e., one resulting from treatment or processing of whole blood to increase the proportion of fetal nucleated cells present, referred to as an enriched maternal sample). An enriched maternal sample is produced, for example, in a two-step process. The maternal sample is subjected to initial separation on the basis of size, such as by Ficoll-Hypaque density gradient centrifugation. This results in production of a supernatant layer, which contains platelets; a mononuclear cell layer; and an agglutinated pellet which contains non-nucleated erythrocytes and granulocytes. The mononuclear layer is separated from the other layers, to produce a maternal sample which is enriched in fetal nucleated cells.

The maternal sample, whether maternal whole blood or an enriched maternal sample, is subjected to separation, based on surface antigenic differences between fetal nucleated cells and maternal cells using antibodies described above. The maternal sample is contacted with at least one monoclonal antibody which is specific for either fetal nucleated cells or maternal cells, but not for both and, thus, makes it possible to separate the two types of cells. The maternal sample can be combined with a set of two or more monoclonal antibodies, each of which is specific for either fetal or maternal cells, but not for both. The combination of monoclonal antibodies can be designed to enhance separation of the two types of cells (e.g., the combination of anti-TfR antibody and HLe-1 antibody described previously) beyond that possible with a single monoclonal antibody. Separation of the fetal cells is carried out using known techniques, such as flow cytometry, use of immunomagnetic beads and cell panning. In general, the monoclonal antibodies have a detectable label (e.g., radioactive material, fluorophore).

An embodiment of the method of the present invention by which fetal cells are isolated and fetal DNA is detected is represented schematically in Figure 1. A maternal blood sample (typically 20 ml.) is obtained, using known techniques. The sample is separated into component layers on the basis of size and the mononuclear cell layer, referred to as the maternal sample enriched in nucleated cells (or enriched maternal sample), is removed for further processing. The enriched maternal sample is contacted with at least one monoclonal antibody, as described above, and the resulting fetal nucleated cell/antibody complexes are separated using known methods (e.g., flow cytometry, immunomagnetic beads, cell panning). Fetal DNA is crudely extracted from the resulting complexes (e.g., by heat), thus rendering it available for hybridization with nucleic acid probes. Fetal DNA can be analyzed for a selected DNA sequence or DNA sequences, using known techniques. Prior to analysis, fetal DNA can be amplified, as needed, using known methods (e.g., PCR).

If amplification is to be carried out, the sorted samples are amplified for an appropriate number of cycles of denaturation and annealing (e.g., approximately 25-60). Control samples include a tube without added DNA to monitor for false positive amplification. With proper modification of PCR conditions, more than one separate fetal gene can be amplified simultaneously. This technique, known as "multiplex" amplification, has been used with six sets of primers in the diagnosis of DMD (Chamberlain, J.S., et al., Prenat. Diagnosis, **9**:349-355 (1989)). When amplification is carried out, the resulting amplification product is a mixture which contains amplified fetal DNA of interest (i.e., the DNA whose occurrence is to be detected and/or quantitated) and other DNA sequences. The amplified fetal DNA of interest and other DNA sequences are separated, using known techniques. Subsequent analysis of amplified DNA can be carried out using known techniques, such as: digestion with restriction endonuclease, ultraviolet light visualization of ethidium bromide stained agarose gels, DNA sequencing, or hybridization with allele specific oligonucleotide probes (Saiki, R.K., et al., Am. J. Hum. Genet., **43** (Suppl):A35 (1988)). Such analysis will determine whether polymorphic differences exist between the amplified "maternal" and "fetal" samples. In one embodiment, the amplification mixture is separated on the basis of size and the resulting size-separated fetal DNA is contacted with an appropriate selected DNA probe or probes (DNA sufficiently complementary to the fetal DNA of interest that it hybridizes to the fetal DNA of interest under the conditions used). Generally, the DNA probes are labelled (e.g., with a radioactive material, a fluorophore or other detectable material). After the size-separated fetal DNA and the selected DNA probes have been maintained for sufficient time under appropriate conditions for hybridization of complementary DNA sequences to occur, resulting in production of fetal DNA/DNA probe complexes, detection of the complexes is carried out using known methods. For example, if the probe is labelled, fetal DNA-labelled DNA probe complex is detected and/or quantitated (e.g., by autoradiography, detection of the fluorescent label). The quantity of labelled complex (and, thus, of fetal DNA) can be determined by comparison with a standard curve (i.e., a predetermined relationship between quantity of label detected and a given read-

ing).

The present method has been used to identify Y-specific DNA in nucleated erythrocytes obtained from peripheral blood of pregnant women. This is described in Example 3. Briefly, candidate fetal cells from blood samples obtained from 19 pregnant women were isolated by flow sorting. The DNA in these cells was amplified for a 222 base pair (bp) sequence present on the short arm of the Y chromosome as proof that the cells were derived from the fetus. The amplified DNA was compared with standardized DNA concentrations; 0.1 to 1 ng fetal DNA was obtained in the 20 ml maternal samples. In 7/19 cases, a 222 bp band of amplified DNA was detected, consistent with the presence of male DNA in the isolated cells; 6/7 of these were confirmed as male pregnancies by karyotyping amniocytes. In the case of the female fetus, DNA prepared from cord blood at delivery also showed the presence of the Y chromosomal sequence. In 10/12 cases where the 222 bp band was absent, the fetuses were female. Thus, the Y chromosomal sequence was successfully detected in 75% of the male-bearing pregnancies, demonstrating for the first time that it is possible to isolate fetal gene sequences from maternal blood.

As described in Example 6, male (Y-specific) DNA has been detected in cells sorted from pregnant women at various points in gestation. Briefly, the mononuclear cell layer was isolated from venous blood samples obtained from women between 11 and 16 weeks gestation. Separation was carried out using Ficoll/Hyque density centrifugation, followed by incubation with monoclonal antibodies (Anti-TfR, anti-Leu 4 and anti-Leu^{M3}) conjugated with a fluorescent marker or compound (fluorescein, phycoerythrin) and dual color analysis and flow sorting on a fluorescence-activated cell sorter. The cells that displayed green fluorescence, but not red fluorescence (TfR positive, Leu 4 negative, Leu M3 negative), were fetal nucleated cells and were separated from the remainder of the sample. These cells were lysed, after which the DNA was amplified and probed for the presence of a 397 bp sequence of the Y chromosome.

The results presented in Example 6 indicate the procedure allows the detection of the 397 bp sequence present in as little as 5 pg of male DNA. In addition, they suggest that there is a relationship between gestational age and detection of male DNA, as illustrated in Figure 4. This data suggests there may be a biologic "window" for transfer of fetal nucleated erythrocytes into maternal circulation.

The present method also has been used to distinguish female fetal DNA from maternal DNA. The two types of female DNA were distinguished using amplification of paternal polymorphism, as described in detail in Example 7. Briefly, venous blood samples were collected from women with uncomplicated pregnancies. Separation of fetal nucleated cells was conducted using Ficoll/Hyque density centrifugation, followed by incubation with monoclonal antibodies (anti-TfR, anti-Leu 4 and anti-Leu M3) conjugated with a fluorescent marker (fluorescein, phycoerythrin) and dual color analysis and flow sorting on a fluorescence-activated cell sorter. Fetal nucleated cells identified by displaying green fluorescence (TfR positive), but not red fluorescence (Leu-4, Leu-3 negative), were collected and lysed. The DNA from the cells was amplified and probed for paternal sequences of the highly polymorphic region of chromosome 17, which allows the distinction of female fetal DNA from maternal DNA.

The results demonstrated that DNA sequences from the father can be identified in the autosomal chromosomes of the fetus. Consequently, the method of the present invention can be used to separate female fetal nucleated cells, as well as male fetal nucleated cells, from maternal blood. Thus, the method can be used for all DNA-based diagnostic procedures currently being used in other methods, such as amniocentesis.

Further support for the present method's capability to identify Y-specific DNA in nucleated erythrocytes obtained from peripheral blood of pregnant women is given by reconstruction experiments. As described in Example 8, male cord blood was added to blood obtained from non-pregnant females to simulate the presence of fetal cells in maternal blood. Briefly, venous blood samples were collected from healthy, non-pregnant women and the mononuclear cell layers isolated by Ficoll/Hyque density centrifugation. Mononuclear cells from the umbilical cords of male infants (ranging from 10² to 10⁶ cells) were added to the mononuclear cell layers of the blood of non-pregnant women. The cord blood contains a large percentage of nucleated erythrocytes. The results obtained from these experiments were substantially similar to those obtained from pregnant women at various stages in gestation. Amplified sequences from the Y chromosome, consistent with the presence of male DNA, were detected when 10² male cells were added to the female cells.

The results of the work described above and in the Examples demonstrate that nucleated fetal cells have been isolated from maternal blood; genomic DNA has been extracted from the fetal cells and identified as being of fetal origin; fetal genes have been amplified using PCR; and selected DNA sequences have been identified in the fetal DNA. They demonstrate that for the first time, fetal DNA has been detected in cells isolated from maternal blood.

Uses of the Present Method of Fetal Nucleated Cell Isolation and Fetal DNA Characterization

Thus, it has been demonstrated that fetal DNA can be obtained from fetal nucleated cells present in a maternal blood sample. The method of detecting and/or quantitating fetal DNA which is represented in Figure 1 is useful as a tool for prenatal assessment (e.g., as a means for assessing chromosomal abnormalities, for determining whether DNA associated with a disease is present, or for detecting Y-specific DNA). It is particularly useful because it is noninvasive and requires only a small sample of blood.

Fetal DNA sequences in fetal nucleated erythrocytes, isolated as described herein or by other means by which fetal nucleated cells can be separated from a maternal blood sample, can be analyzed or assessed for the occurrence of a DNA sequence or DNA sequences (gene(s) or gene portion(s)) which are of interest for diagnostic or other purposes. The DNA sequence(s) or gene(s)/gene portion(s) present in fetal cells are referred to herein as fetal DNA of interest.

5 For example, the selected DNA whose presence or absence is to be determined and whose quantity can also be determined is the gene for a disease, such as cystic fibrosis, where the causative gene or gene portion has been cloned and sequenced; alternatively, it is a probe for X- or Y-specific DNA. The same procedure can also be used, with appropriate modifications (e.g., an appropriate DNA probe, time, temperature), to detect other genes or gene portions.

As used in a diagnostic context, such as to detect the gene known to cause cystic fibrosis, the present method is carried out as follows: Initially, a maternal blood sample (typically 20 ml.) is obtained and separated into component layers based on relative weights (e.g., by Ficoll-Hypaque density gradient centrifugation) to remove non-nucleated erythrocytes and produce a mononuclear cell layer. This results in production of a maternal blood sample enriched in fetal nucleated erythrocytes. The mononuclear cell layer is stained with at least one appropriate monoclonal antibody (e.g., one which is specific for the type of fetal nucleated cell to be separated from the sample). For example, a monoclonal antibody specific for fetal nucleated cells, such as anti-TfR antibody, described above, can be used. In general, the monoclonal antibody used bears a detectable label. Alternatively, a combination of selected labelled monoclonal antibodies, such as monoclonal antibodies specific for fetal nucleated cells (e.g., anti-TfR antibody) and monoclonal antibodies specific for maternal leucocytes (HLe-1 or L4 and M3), each labelled with a different fluorescent compound, can be used to remove essentially all maternal cells. Labelled cells are subsequently separated from one another using a known method, such as flow cytometry. Binding of the monoclonal antibodies to cells for which they are specific results in production of labelled monoclonal antibody-cell complexes. For example, in the case in which anti-TfR antibodies and HLe-1 are used, fetal nucleated erythrocytes are bound by anti-TfR antibody, to produce fetal nucleated erythrocytes/anti-TfR antibody complexes, and maternal leucocytes are bound by HLe-1 antibodies, to produce maternal leucocyte/HLe-1 antibody complexes. The fetal nucleated erythrocyte/anti-TfR antibody complexes are separated from maternal cell/HLe-1 antibody complexes, using, for example, flow cytometry. The fetal cells are lysed, to produce crudely extracted fetal DNA which is subsequently amplified, using, for example, PCR. This results in production of amplified fetal DNA, which is subsequently separated on the basis of size. Size-separated fetal DNA is contacted with labelled DNA probes (i.e., in prenatal detection of cystic fibrosis, a labelled DNA probe complementary to the gene associated with cystic fibrosis). If the fetal DNA contains DNA of interest (in this case, the gene associated with cystic fibrosis), fetal DNA of interest/labelled probe complexes are formed.

Fetal DNA of interest/labelled probe complexes are subsequently detected, using a known technique, such as autoradiography. Simple presence or absence of labelled fetal DNA of interest can be determined or the quantity of fetal DNA of interest present can be determined. In either case, the result is assessment of fetal DNA obtained from a maternal blood sample for selected DNA.

The occurrence of fetal DNA associated with diseases or conditions other than cystic fibrosis can also be detected and/or quantitated by the present method. In each case, an appropriate probe is used to detect the sequence of interest. For example, sequences from probes St14 (Oberle, I., et al., *New Engl. J. Med.*, **312**:682-686 (1985)), 49a (Guerin, P., et al., *Nucleic Acids Res.*, **16**:7759 (1988)), KM-19 (Gasparini, P., et al., *Prenat. Diagnosis*, **9**:349-355 (1989)), or the deletion-prone exons for the Duchenne muscular dystrophy (DMD) gene (Chamberlain, J.S., et al., *Nucleic Acids Res.*, **16**:11141-11156 (1988)) are used as probes. St14 is a highly polymorphic sequence isolated from the long arm of the X chromosome that has potential usefulness in distinguishing female DNA from maternal DNA. It maps near the gene for Factor VIII:C and, thus, may also be utilized for prenatal diagnosis of Hemophilia A. Primers corresponding to sequences flanking the six most commonly deleted exons in the DMD gene, which have been successfully used to diagnose DMD by PCR, can also be used (Chamberlain, J.S., et al., *Nucleic Acids Res.*, **16**:11141-11156 (1988)). Other conditions which can be diagnosed by the present method include β-thalassemia (Cai, S-P., et al., *Blood*, **73**:372-374 (1989); Cai, S-P., et al., *Am. J. Hum. Genet.*, **45**:112-114 (1989); Saiki, R.K., et al., *New Engl. J. Med.*, **319**:537-541 (1988)), sickle cell anemia (Saiki, R.K., et al., *New Engl. J. Med.*, **319**:537-541 (1988)), phenylketonuria (DiLella, A.G., et al., *Lancet*, **1**:497-499 (1988)) and Gaucher disease (Theophilus, B., et al., *Am. J. Hum. Genet.*, **45**:212-215 (1989)). An appropriate probe (or probes) is available for use in the present method for assessing each condition.

It is also possible to separate fetal cells from maternal cells by means other than flow cytometry, as mentioned previously, and to analyze fetal nucleated erythrocyte DNA obtained in this way. Such separation procedures may be used in conjunction with or independent of flow cytometry. This is advantageous because lack of access to a flow cytometer, as well as expense, could limit potential applications of this technique. Thus, other methods of fetal cell separation can be used. The separation method used can result in elimination of unwanted cells ("negative selection") or isolation of rare but desirable cells ("positive selection").

For example, separation by immunomagnetic beads or by cell panning can be used. In this embodiment, the mononuclear cell layer is isolated, as described previously. This layer is then mixed with antibody-coated polymer particles containing magnetic cores (e.g., "Dynabeads"). These immunomagnetic beads are available coated with a variety of antibodies. For example, immunomagnetic beads coated with antibody to leucocyte antigens and antibody to mouse

immunoglobulins, which can be subsequently conjugated to mouse monoclonal antibody against the human transferrin receptor, can be used. After mixing, the rosetted cells are isolated with a magnetic particle concentrator. In one embodiment, two sets of antibody-coated immunomagnetic beads are used in succession. First, the maternal leucocytes are depleted and then the remaining TfR positive cells are collected. Subsequent steps in the method (amplification, separation, contact with an appropriate DNA probe or probe set) are as described for cells separated by flow cytometry.

Mueller *et al.* (*Lancet*, **336**: 197-200 (1990)) have described a method of isolating placenta-derived trophoblast cells in the blood of pregnant women using magnetic beads. This method included mixing 1 ml of monoclonal antibody hybridoma culture supernatant with 2×10^7 magnetic beads precoated with sheep antibody to mouse IgG (Fc fragment) (Dynabeads M-450, Dynal AS, Oslo, Norway) and incubated overnight at room temperature. The coated beads were stored at 4°C and washed three times in ice-cold RPMI 1640 medium containing lithium heparin (10 IU/ml). The blood from the pregnant women was collected into tubes containing 10 IU of lithium per ml of whole blood, diluted 1:10 with RPMI containing lithium, and incubated with the antibody coated beads at 4°C overnight. The desired cells were bound to the antibody on the bead; the beads collected by means of a cobalt-samarium magnet. Although in this case the antibody was directed against trophoblast antigens, a similar technique can be utilized with, for example, antibody to cell surface antigens present on fetal nucleated erythrocytes and not present on maternal cells. An advantage to this particular technique is that an initial step which results in mononuclear cell isolation is not added. Additionally, the magnetic beads can be used for both positive (fetal cells) and negative (maternal cells) selection.

An alternative method of isolation can be a modification of the method described by R.J. Berenson *et al.* (*J. of Immunol. Methods*, **91**: 11-19 (1986)) in which the high affinity between the protein avidin and the vitamin biotin was exploited to create an indirect immunoabsorptive procedure. In this technique, avidin was linked to cyanogen bromide activated sepharose 6MB beads and washed in an alternating fashion with coupling buffer (0.1 M NaHCO₃ in 0.5 M NaCl at pH 8.3) and washing buffer (0.1 M sodium acetate in 0.5 M NaCl at pH 4.5) and stored at 4°C. The blood cells were incubated with 1) murine monoclonal antibody, and 2) biotinylated goat anti-mouse immunoglobulin. A 3 ml column of gel was packed in a Pharmacia K 19/15 column. The treated cells were passed through the column in phosphate buffered saline containing 2% bovine serum albumin. Adherent cells were dislodged by mechanical agitation. This technique can be applied to fetal cell separation if the antibodies used recognize fetal cell surface antigens or maternal cell surface antigens, but not both. Variations in methods for conjugating antibodies to beads exist; examples include those described by Thomas and co-workers (Thomas, T.E., *et al.* (*J. of Immuno. Methods*, **120**: 221-131 (1989)) and by deKretser and co-workers (deKretser, T.A., *et al.* (*Tissue Antigens*, **16**: 317-325 (1980)). The use of an antibody-bound column does not require the preliminary isolation of the mononuclear cell fraction from whole blood.

Once the fetal cells are isolated from maternal blood, they may be cultured to increase the numbers of cells available for diagnosis, if desired. E. Fibach *et al.* (*Blood*, **73**: 100-103 (1989)) have described a method that supports the growth of human hematopoietic progenitor cells. This step-wise method involves 1) initial culture in the presence of conditioned medium from human bladder carcinoma cells, 2) removal of leucocytes by harvest of non-adherent cells and lysis with monoclonal antibodies, and 3) reculture of cells in medium supplemented by recombinant erythropoietin.

Other methods of separating fetal nucleated cells from maternal cells can also be used, provided that they make it possible to differentiate between fetal cells and maternal cells, and to isolate one from the other.

A kit for use in carrying out the present method of isolating and detecting fetal DNA of interest, such as a chromosomal abnormality associated with a disease or other condition, in a maternal blood sample can be produced. It includes, for example, a container for holding the reagents needed; the reagents and, optionally, a solid support for use in separating fetal nucleated cell-specific antibody complexes from other sample components or for removing maternal cells complexed with specific antibody. For example, reagents in a kit to be used in detecting fetal DNA of interest after amplification of fetal DNA by PCR can include: 1) at least one antibody specific for a surface antigen characteristic of fetal nucleated cells but not specific for a surface antigen characteristic of maternal leucocytes; selected DNA primers for use in amplifying fetal DNA by PCR; and at least one DNA probe complementary to the fetal DNA to be detected (fetal DNA of interest). The kit, as indicated, can also include a solid support to be used in separating complexes formed from other samples components. Such solid support can be, for example, a glass slide, nitrocellulose filter, or immunomagnetic beads and can have affixed thereto an antibody selective for the antibody present in the fetal nucleated cell-specific antibody complexes.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLE 1 Antibody selection for isolation and sorting of fetal nucleated erythrocytes (NRBCs)

55 Removal of maternal leucocytes from maternal blood using human leucocyte antigen (HLe-1)

The technique of fetal NRBC isolation began with an initial Ficoll-Hypaque density gradient centrifugation to remove the tremendously high number of non-nucleated erythrocytes in maternal blood. Peripheral blood was centrifuged and separated into a supernatant layer containing platelets, a mononuclear cell layer, and an agglutinated pellet

consisting of non-nucleated erythrocytes and granulocytes. The mononuclear cell layer consisted of lymphocytes, monocytes, possible trophoblasts, and, due to their increased size and density, NRBCs and some reticulocytes. While the Ficoll-Hypaque centrifugation represented an initial enrichment in the proportion of fetal NRBCs present in the maternal sample, flow cytometry and cell sorting was used to improve the purity of the isolated cell population.

5 The mononuclear cell layer from peripheral blood samples in 63 pregnant women, 15 nonpregnant adults, and 39 umbilical cords, was stained with FITC-HLe-1 for flow cytometric analysis. Umbilical cord samples were used as a substitute for whole fetal blood. Representative histograms displaying fluorescence versus low-angle light scatter (an approximation of cell size) for each of the three groups were generated. Histogram peaks were identified that corresponded to leucocytes, erythrocytes and platelets. In 9 pregnant women, 7 nonpregnant adults and 12 umbilical cord
10 samples, fluorescent (HLe-1 positive) and non-fluorescent (HLe-1 negative) cell populations were sorted for detailed microscopy after Wright-Giemsa staining. While the HLe-1 positive populations were always composed of leucocytes independent of the sample source, the HLe-1 negative populations differed.

In cord blood, the HLe-1 negative cells were non-nucleated and nucleated erythrocytes with occasional platelets. In the pregnant women, there were platelets, non-nucleated erythrocytes, and a very rare NRBC. In non-pregnant
15 adults, only platelets and debris were seen. Thus, cord blood, with its high percentage of NRBCs, was used as a reference to establish cell sorting parameters. Microscopy confirmed the specificity of the antibody-antigen binding and that the sorted HLe-1 negative cells were relatively free from leucocyte contamination. These sorting parameters were utilized to isolate potential fetal NRBC on 40 pregnancies.

20 Enrichment of fetal NRBC in maternal blood using transferrin receptor antigen (TfR)

The transferrin receptor (Newman, R., et al., *Trends Biochem. Sci.* 1:397-399 (1982)) is a surface glycoprotein important in cellular iron transport. The TfR is present on activated lymphocytes (Trowbridge, I.S., et al., *Proc. Natl. Acad. Sci. USA*, 78:3039-3043 (1981)), certain tumor cells (Greaves, M., et al., *Int. J. Immunopharmac.*, 3:283-300 (1981)), and trophoblast cells (Galbraith, G.M.P., et al., *Blood*, 55:240-242 (1980)). Erythroblasts express the TfR on their cell surfaces from the BFU-E stage until nuclear extrusion (Loken, M.R., et al., *Blood*, 69:255-263 (1987)). Thus, TfR is an excellent "candidate antigen" for enrichment of fetal NRBCs found in maternal blood. Monoclonal antibody against TfR is available as both a fluorescein conjugate (Becton-Dickinson catalog #7513) and a phycoerythrin (PR) conjugate (gift of Dr. Michael Loken, Becton-Dickinson). The mononuclear cell layer was isolated from peripheral blood
25 samples in 6 pregnant women, 4 non-pregnant adults, and 3 newborn umbilical cords for TfR analysis and microscopy. Representative histograms of fluorescence versus light scatter from these three groups were generated.

Whereas umbilical cord samples had a large population of fluorescent (TfR positive cells) that were heterogenous in size, non-pregnant adults and pregnant adults had smaller percentages of fluorescent cells that clustered in discrete groups. In addition, there were slight differences in the percentages of TfR positive cells in the pregnant (mean = 0.83)
30 versus non-pregnant (mean = 0.32) samples studies.

Microscope studies of the TfR positive cells were performed using Wright-Giemsa stain for morphology and Kleihauer-Betke technique for the detection of fetal hemoglobin (Kleihauer, E., et al., *Klin. Wochenschr.*, 35:637-638 (1957)). In the umbilical cord samples, large numbers of nucleated and non-nucleated erythrocytes containing fetal hemoglobin and occasional leucocytes were identified visually. In the pregnant women, the predominant cell types were
35 nucleated and non-nucleated erythrocytes containing fetal hemoglobin, although leucocytes were infrequently observed. In contrast, the samples from the non-pregnant controls consisted almost exclusively of lymphocytes and monocytes. Because trophoblast cells express TfR, it was postulated that they might be present in the sorted population from the pregnant women; none was detected.

40 Dual antibody analysis

Because both antibodies enriched the proportion of NRBCs present, but did not completely exclude other cell types in the sorted samples, combinations of antibodies were used to isolate pure populations of fetal NRBCs. Preliminary dual antibody studies were performed using PE-conjugated TfR and FITC-conjugated HLe-1. NRBCs are TfR positive
45 and HLe-1 negative, whereas maternal leucocytes are HLe-1 positive. These experiments worked well and resulted in separation of maternal leucocytes.

Thus, the work described above defined flow cytometric parameters for enrichment and sorting of NRBCs in peripheral blood from pregnant women. In addition, microscopic studies revealed that morphologic differences occur in mononuclear cell populations derived from venous blood samples in pregnant versus non-pregnant adults.

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EXAMPLE 2 DNA hybridization studies in HLe-1 negative cells sorted from maternal blood

To confirm fetal origin of the cells sorted as described in Example 1, Y chromosomal probes were used because it is the Y chromosome that is unquestionably fetal in origin. The assessments were designed to study whether the pres-

ence of Y chromosomal DNA in maternal blood as detected on autoradiographs performed antenatally correlated with the subsequent birth of a male infant.

DNA isolation

HLe-1 negative cells from cord blood and pregnant women were sorted into test tubes. Conventional methods of DNA isolation as well as modification of cruder methods (Lau, Y-F., et al. *Lancet*, 1:14-16 (1984); McCabe, E.R.B., et al., *Hum Genet*, 75:213-216 (1987)) were attempted without success in detecting Y chromosome derived bands on Southern Blots. All were limited by the small numbers of cells present.

EXAMPLE 3 Direct hybridization to cells deposited on filters

In order to circumvent technical problems associated with DNA isolation, a method of direct DNA hybridization to cells flow sorted onto nitrocellulose filters was developed (Bianchi, D.W., et al., *Cytometry*, 8:197-202 (1987)). In control experiments, the sex of a newborn was determined from as few as 50 sorted cord blood leucocytes or 5,000 HLe-1 negative cells (a mixture of nucleated and non-nucleated cells).

The methodology was then applied to detection of Y chromosomal sequences in HLe-1 negative cells sorted from peripheral blood samples in 40 women between 8½ and 38 weeks gestation. Results were the following:

| Dot Blot Hybridization Y Chromosomal Probe | with Delivered Male Infant | Delivered Female Infant | Lost to Follow-up |
|--|----------------------------|-------------------------|-------------------|
| + | 3 | 2 | 0 |
| - | 21 | 12 | 2 |

It was concluded that hybridization with this probe was not predictive of male pregnancy. The possibility exists that there was fetal DNA present on the filters where DNA hybridization occurred, but that this DNA bound to the Y probe nonspecifically. Thus, the filters interpreted as "positive" for male DNA might actually have been "positive" for fetomaternal hemorrhage.

EXAMPLE 4 Use of the polymerase chain reaction (PCR) to amplify gene sequences in sorted fetal cells

PCR, which has a capacity for making 10^6 Copies of rare target gene sequences, was used to amplify gene sequences in sorted fetal cells. Optimum conditions for PCR, given the minute amounts of DNA expected after a fetal cell sort (approximately 1 pg to 100 ng), were determined. Experimental conditions were modified as new information became available. For example, Taq polymerase was used instead of Klenow fragment of *E. Coli* DNA polymerase (Kogan, S.C. et al., *New England J. Med.* 317:990 (1987)) because of its increased specificity in DNA replication.

Initially, studies were performed on repeated sequences from the long arm of the Y chromosome, probe Y431-Hintf (given by Dr. Kirby Smith, Johns Hopkins University, Baltimore, MD) and the short arm of the Y chromosome, probe Y411 (Given by Dr. Ulrich Muller, Children's Hospital, Boston, MA). Repeated sequences were selected because they would create a stronger amplification signal from a rare male fetal cell. Y411 is identical to Y156 (Muller, U., et al., *Nucleic Acids Res.*, 14:1325-1329 (1986)), is repeated 10-60 fold, and is absolutely Y specific on Southern blots. Sequence Y431 has autosomal homology in females that limited its usefulness in sex determination.

PCR standardization

To define the minimum amount of DNA detectable in maternal blood, a series of standardization experiments were done. DNA from male and female individuals was prepared in tenfold dilutions (1 pg to 1 mcg) and amplified using the standard reagents in the GeneAmp kit (Perkin-Elmer Cetus cat #N801-0055) on a Perkin-Elmer DNA Thermal Cycler. Primers 411-01 and 411-03 were designed to amplify a 222 base pair (bp) sequence within probe Y411. The number of amplification cycles varied between 18 and 30. Amplified DNA samples were electrophoresed on agarose gels, transferred to nylon filters, and hybridized to 32 P-labeled Y411 probe. While it appeared possible to detect Y specific bands on autoradiographs in lanes containing as little as 10 pg of male DNA, results were often muddled by the presence of amplified DNA in female lanes or control lanes containing no added DNA. The phenomenon of "false positive amplification" has now received universal recognition (Lo, Y-M.D., et al., *Lancet*, 2:697 (1988); Kwok, S., et al., *Nature*, 339:237-238 (1989)).

Elimination of "false positive" amplification

Due to the limited amount of starting material in a fetal cell sort, every effort was made to eliminate background amplification in order to determine which fetuses truly possess Y chromosomal DNA. Thus, measures were taken to prevent aerosol contamination of male DNA. All PCRs were performed under sterile conditions, wearing gloves, and using positive displacement pipettes. All reagents were prepared in a sterile manner and incubated overnight prior to PCR with a restriction endonuclease having a digestion site within the target sequence. These precautions resulted in a significant decrease and virtual absence of false positive amplification, as monitored by running control reactions with all reagents but no DNA.

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Successful isolation and amplification of fetal gene sequences from NRBCs in maternal blood

After eliminating sources of DNA contamination and determining that as little as 10 pg of male DNA (1 cell = 7 pg of DNA) could be detected after PCR amplification, candidate fetal cells from the peripheral blood of 19 women at 12½ to 17 weeks gestation were sorted. Monoclonal antibody against TfR was used to identify the presumed NRBC. The DNA in the sorted cells was amplified for the 222 bp sequence in probe Y411 as proof that the cells were derived from the fetus in male pregnancies. In 7/19 cases the 222 bp band of amplified DNA was detected on autoradiographs, consistent with the presence of male DNA in the isolated cells; 6/7 of these were confirmed as male pregnancies by karyotyping amniocytes. In the case of one female fetus, repeat studies at 32 weeks gestation and cord blood at delivery also showed the presence of the Y chromosomal sequence. This result might be explained by a low level of sex chromosome mosaicism, XX/XY chimerism (Farber, C.M., et al., *Hum. Genet.*, **82**:197-198 (1989)), or the presence of the Y411 sequence in single copy on the X chromosome or autosomes. In 10/12 cases where the 222 bp was absent, the fetuses were female. Therefore, detection of the Y chromosomal sequence was successful in 6/8 or 75% of the male-bearing pregnancies. In the two pregnancies where male DNA was not detected, there may have been fetomaternal blood group incompatibility. Alternatively, there may not have been fetomaternal hemorrhage or the number of NRBCs present may have been below the limit of sensitivity for detection of DNA. The conditions used made it possible to detect a minimum of 100 pg of fetal DNA, or the equivalent of 15 fetal cells. The limit of sensitivity can be improved by extending the number of cycles used in PCR. This work demonstrated that for the first time, fetal DNA was detected in cells isolated from maternal blood.

30

To further decrease false positive amplification and permit detection of fetal DNA at the single cell level on agarose gels, PCR is being carried out using primers derived from a single copy of sequence specific for the long arm of the Y chromosome, pY49a (Guerin, P., et al., *Nucleic Acids Res.*, **16**:7759 (1988)). In preliminary experiments using 60 cycles of PCR, Y chromosomal DNA is visible on ethidiumbromide stained agarose gels. This extraordinary degree of sensitivity will now be applied to DNA from sorted fetal cells.

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EXAMPLE 5 Determination of the Volume, Morphology and Universality of Fetomaternal Hemorrhagea. General Strategy

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It is also possible, because of the availability of the present method of isolating fetal nucleated cells from blood obtained from a pregnant woman, to determine whether fetal cells can be found in the maternal blood in all pregnancies. A data base can be created that can provide information on the number and type of fetal cells circulating in maternal blood as pregnancy progresses. Based on previous work, it is anticipated that there will be a normal range of values that is dependent on gestational age; deviation from these values will be studied as a potential indication of a pregnancy at risk. Specifically, large amounts of fetal blood in the maternal circulation may be correlated with placental abnormalities, threatened miscarriage and intrauterine growth retardation.

45

Maternal venous blood samples are collected from pregnant women, generally prior to any invasive procedures. In general, a single 20 ml. venous blood sample will be obtained. In a subgroup of patients, permission will be sought to draw blood samples every 4 weeks to follow changes in numbers of fetal cells present. Blood is collected in EDTA, diluted 1:1 with Hanks Balanced Salt Solution (HBSS), layered over a Ficoll-Hypaque column (Pharmacia) and spun at 1400 rpm for 40 minutes at room temperature. The mononuclear cell layer will be isolated, washed twice with HBSS, and stained with fluorescent monoclonal antibodies. For example, this can be a combination of fluorescein isothiocyanate-conjugated antitransferrin receptor (TfR) and phycoerythrin-conjugated anti-monocyte antibodies (M3, Becton-Dickinson catalog #7497) and anti-lymphocyte antibodies (L4, Becton-Dickinson catalog #7347). The staining occurs on ice, in phosphate buffered saline (PBS) containing 2% fetal calf serum and 0.1% sodium azide. The cells are washed in PBS prior to flow cytometry. Analysis and sorting are performed on a Becton-Dickinson FACS-IV interfaced with a Consort 40 program. Data will be acquired on the relative size and fluorescence (in two colors) of the analyzed cells. Cells that are fluorescent in the green wavelength (TfR positive) and not fluorescent in the red wavelength (L4 and M3 negative) will contain the presumed fetal NRBCs. The percentage of these cells in the mononuclear cell layer are-

corded and analyzed as a function of gestational age. These cells are sorted for microscopy and PCR amplification. In addition, cells that are not fluorescent in the green wavelength (TfR negative) but are fluorescent in the red wavelength (L4 and/or M3 positive) are sorted as a presumed maternal leucocyte population and source of maternal DNA polymorphisms.

5 An additional benefit of studying nucleated fetal cells in maternal blood is that the amount of fetal DNA present can be extrapolated to determine the extent of fetomaternal hemorrhage in normal and unusual pregnancies. In the pregnancies studied, an average amount of 1 ng of fetal DNA (corresponding to 150 NBCRs) was present. Using published values of the number of NRBCs per liter of fetal blood at 16 weeks (3.6×10^9) (Millar, D.S., et al., *Prenat. Diagnosis*, 5:367-373 (1985); (Forestier, F., et al., *Pediatr. Res.*, 20:342-346 (1986)) and doing simple algebra, these results were
10 calculated to be consistent with 2-20 μ l hemorrhage of fetal blood into maternal circulation. This is a trivial amount when compared with the fetoplacental blood volume at 16 weeks, about 20 ml. It is important to validate and extend these results to generate normative data regarding fetomaternal transfusion in early pregnancies. It will be equally important to correlate deviations from the expected results with pregnancy complications.

15 Example 6 Detection of Male DNA in Cells Sorted from Pregnant Women at Different Points in Gestation

Venous blood samples (20 ml) were collected in EDTA from healthy women with uncomplicated pregnancies, prior to invasive diagnostic procedures, at different points in gestation. The mononuclear cell layer was isolated by Ficoll/Hypaque density centrifugation and incubated with the monoclonal antibodies fluorescein (FITC)-conjugated
20 anti-TfR, phycoerythrin (PE)-conjugated anti-Leu 4 and PE-conjugated anti-Leu M3 (Becton-Dickinson). Dual color analysis and flow sorting were performed on a fluorescence-activated cell sorter.

Cells that display green fluorescence but not red fluorescence (TfR positive, Leu 4 negative, Leu M3 negative) were collected into sterile micro test tubes and frozen at -20°C. Prior to polymerase chain reaction amplification, the cells were lysed by boiling. The polymerase chain reaction (PCR) was performed under standard conditions using standard
25 reagents as described in Example 4. The primers used to amplify material from the Y chromosome define a 397 base pair (bp) sequence. After PCR, the patient samples were analyzed with conventional Southern blots using 32 P labelled probe. Ethidium bromide stained agarose gels and autoradiographs were examined for the presence of the 397 bp band, which is considered significant only if reagent controls do not reveal false positive amplification.

Under the reaction conditions described above, it was possible to detect the 397 bp male specific band if 5 pg of
30 male DNA was present. This is approximately the amount of DNA present in one cell. When excess female DNA (500 ng) was added to the reaction mixture, the male specific band was consistently detectable at 100 pg.

Figure 4 represents a summation of samples obtained from twelve women bearing male fetuses. These samples were taken at different times in pregnancy, and one woman was sampled twice. The data indicates that there is a relationship between gestational age and the detection of male DNA. This implies a potential biologic "window" for the
35 transfer of fetal nucleated erythrocytes into the maternal circulation.

Example 7 Detection of Female Fetal DNA by Amplification of Paternal Polymorphisms

Venous blood samples (20 ml) were collected in EDTA from healthy women with uncomplicated pregnancies. The
40 mononuclear cell layer was isolated by Ficoll/Hypaque density centrifugation and incubated with the monoclonal anti-bodies fluorescein (FITC)-conjugated anti-TfR, phycoerythrin (PE)-conjugated anti-Leu 4 and PE-conjugated anti-Leu M3 (Becton-Dickinson). Dual color analysis and flow sorting were performed on a fluorescence-activated cell sorter.

Cells that display green fluorescence but not red fluorescence (TfR positive, Leu 4 negative, Leu M3 negative) were collected into sterile micro test tubes and frozen at -20°C. Additionally, cells that displayed red fluorescence but not
45 green fluorescence (TfR negative, Leu 4 positive, Leu M3 positive) were collected in an identical manner. Prior to polymerase chain reaction (PCR) amplification, the cells were lysed by boiling. PCR was performed using buffers containing 1 mM MgCl₂. The primers used in PCR amplify a highly polymorphic region of chromosome 17. Amplified DNA sequences correspond to blocks of genes transmitted directly from parent to child. As a result of the high degree of individual variation in these sequences, it is uncommon for two parents to manifest identical DNA patterns. Thus, it is possible to demonstrate inheritance of the paternal sequences in the sorted fetal cells. Since these sequences are from
50 chromosome 17, they are independent of fetal sex, and may be used to distinguish female fetal DNA from maternal DNA. Amplified DNA was separated by electrophoresis through ethidium bromide stained agarose gels. The DNA was transferred to nylon filters and probed using 32 P labeled sequence. The maternal DNA, paternal DNA, TfR⁺ cells, and TfR⁻ cells were then compared.

55 In 5 of 10 pregnant women, it was possible to show the presence of paternal sequences in the sorted candidate fetal cell population. In the other 5 women, no differences were seen between the maternal DNA and the DNA obtained from the candidate fetal cells.

Example 8 Reconstruction Experiments Using Non-Pregnant Female Blood and Added Male Cord Blood to Simulate the Presence of Fetal Cells in Maternal Blood

Venous blood samples (20 ml) were collected in EDTA from healthy non-pregnant women. Umbilical cord blood samples (10 ml) were collected in EDTA from normal newborns. The mononuclear cell layer was isolated by Ficoll/Hyque density centrifugation. Cell counts were performed with a hemocytometer. Separate aliquots of cells were made containing: 1) female cells alone; 2) female cells plus 10^2 added male cord blood cells; 3) female cells plus 10^3 added male cord blood cells; 4) female cells plus 10^4 added male cord blood cells; 5) female cells plus 10^5 added male cord blood cells; 6) female cells plus 10^6 added male cord blood cells; 7) male cord blood cells alone. The separate aliquots were then incubated with the individual monoclonal antibodies being tested. Analysis and sorting were performed using a flow cytometer. For each aliquot, a bivariate histogram was obtained, and gating parameters were established for antibody positive and antibody negative cells. The sorted cells were collected into sterile micro test tubes and frozen at -20°C. PCR amplification was performed with primers that detect a 397 bp sequence unique to the Y chromosome. The presence of a band at 397 bp in autoradiographs was used to confirm the presence of male umbilical cord blood cells in sorted samples.

Figure 5 shows the histograms obtained when FITC-anti transferrin receptor is used. In the non-pregnant female, 0.1% of the mononuclear cells react with the antibody. In male cord blood, 24.9% of the mononuclear cells react with the antibody. With the addition of more and more umbilical cord cells to the non-pregnant female cells, an increased percentage of cells that react with the antibody is seen.

Figure 6 shows that male DNA is detected in the TfR⁺ cells when 10^2 - 10^6 male cells are added. Male DNA is detected in the TfR⁻ cells when 10^5 - 10^6 male cells are added. This results from the presence of male white blood cells in the TfR⁻ population.

Figure 7 shows the histograms obtained when anti HPCA-1 antibody is used. In the non-pregnant female, 0.9% of the mononuclear cells react with antibody. In umbilical cord blood, a well-defined population of cells is seen, but the percentage is only 1.1%. Thus, the addition of umbilical cord blood cells to the non-pregnant female cells is not seen on the histograms as clearly as with the transferrin receptor antibody. An increased number of HPCA⁺ cells were collected as the amounts of added cord blood cells increased.

In agarose gels, the 397 bp band consistent with DNA was detected in the HPCA⁺ cells when 10^3 - 10^5 male cells were added to the female cells. Male DNA was detected in agarose gels in the HPCA cells when 10^6 male cells were added to the female cells.

Example 9 In situ Hybridization Using Molecular Probes Recognizing Individual Chromosomes in Flow Sorted Nucleated Erythrocytes

To demonstrate diagnostic utility of the present invention, a DNA probe set was constructed of chromosome specific probes that provided both good signal to noise ratios and good spatial resolution of the fluorescent signals. Accordingly, specific probes were developed for five chromosomes frequently seen as liveborn aneuploidies; chromosomes 13, 18, 21, X and Y. A probe for chromosome 1 was used as a control. In constructing the probes, the general strategy was to identify a starting clone that mapped to the desired chromosomal region by multiple genetic and physical methods, and then to use that clone to identify a matching cosmid "contig" which was then used as a hybridization probe.

Hybridization of the high copy number repeat sequences was suppressed by inclusion of total genomic human DNA, and the chromosomal specificity verified by hybridization to metaphase spreads. The probes gave sharp, punctate fluorescent signals in interphase cells that was easily discriminated and enumerated. The Y probe used in this study was pDP97, a repetitive clone (a 5.3 kb EcoRI Y fragment from cosmid Y97 subcloned into EcoRI site of pUC-13). All probes were labeled with biotin, hybridized under suppression conditions, and specific hybridization detected by conjugated streptoavidin-FITC, which showed as a single "dot" in the FITC image. As illustrated in Figure 8, the Y chromosome was detected by *in situ* hybridization of the pDP97 probe for the Y chromosome in a fetal nucleated red blood cell. Thus, prenatal diagnosis for chromosomal abnormalities could be performed on fetal cells isolated from maternal blood.

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Claims

1. A method of separating fetal nucleated cells (e.g. fetal nucleated erythrocytes) present in a sample of blood obtained from a pregnant woman, comprising separating fetal nucleated cells present in the sample of blood from other cells present in the blood on the basis of an antigen present on fetal nucleated cells, or present on other cells present in the blood, but not present on both.
2. An *in vitro* method of separating fetal nucleated erythrocytes present in the blood of a pregnant woman, comprising sorting fetal nucleated erythrocytes present in the blood on the basis of an immunologic feature which is character-

istic of fetal nucleated erythrocytes or characteristic of cells other than fetal nucleated erythrocytes, but not characteristic of both.

3. A method of separating fetal nucleated cells present in a sample of whole blood obtained from a pregnant woman, comprising contacting the sample of whole blood with at least one antibody which is specific for an antigen present on fetal nucleated cells, but not present on maternal cells, under conditions appropriate for binding of the antibody with the antigen present on fetal nucleated cells, thereby forming antibody-fetal nucleated cell complexes and separating antibody-fetal nucleated cell complexes from the sample.
- 10 4. A method of separating fetal nucleated erythrocytes present in a maternal blood sample, comprising the steps of:
 - a) Contacting the blood sample with 1) a first monoclonal antibody, which is specific for an antigen present on fetal nucleated erythrocytes but not for maternal leucocytes and 2) a second monoclonal antibody, which is specific for an antigen present on maternal leucocytes but not on fetal nucleated erythrocytes, under conditions appropriate for binding of monoclonal antibody to specific antigen, thereby producing fetal nucleated erythrocyte/first monoclonal antibody complexes and maternal leucocyte/second monoclonal antibody complexes; and
 - b) separating fetal nucleated erythrocyte/first monoclonal antibody complexes from maternal leucocyte/second monoclonal antibody complexes.
- 20 5. The method of Claim 4 wherein the first monoclonal antibody and the second monoclonal antibody are fluorescently labelled, each with a different fluorescent material and separation in step (b) is by flow cytometry.
- 25 6. A method of separating fetal nucleated erythrocytes present in a sample of blood obtained from a pregnant woman, comprising the steps of:
 - a) combining the maternal sample with at least one detectable monoclonal antibody selective for fetal nucleated erythrocytes, under conditions appropriate for binding of detectable monoclonal antibody with fetal nucleated erythrocytes, to produce bound detectable monoclonal antibody; and
 - b) separating bound detectable monoclonal antibody from the sample.
- 30 7. The method of Claim 6 further comprising,
 - a) in step (a), combining the maternal sample with at least two detectable monoclonal antibodies, wherein the first of said detectable monoclonal antibodies is selective for antigens present on mature human leucocytes and for antigens present on very immature erythrocyte precursors, but not for mature nucleated erythrocytes; the second of said detectable monoclonal antibodies is selective for an antigen which is present on the surface of fetal nucleated erythrocytes; the second of said detectable monoclonal antibodies is selective for an antigen which is present on the surface of fetal nucleated erythrocytes, but not for antigens present on mature human leucocytes or antigens present on very immature erythrocytes; and the two detectable monoclonal antibodies are separately detectable, to produce a first bound detectable monoclonal antibody and a second bound detectable monoclonal antibody; and
 - b) in step (b), separating the first bound detectable monoclonal antibody and the second bound detectable monoclonal antibody from one another; and for example wherein the first of said detectable monoclonal antibodies is selected from the group consisting of HLe-1, L4 and M3 HLe-1 and the second of said detectable monoclonal antibodies is anti-TfR antibody.
- 40 8. A method of separating fetal nucleated cells present in the blood of a pregnant woman, comprising the steps of:
 - a) separating a sample of blood obtained from the pregnant woman into constituent layers, said constituent layers including a mononuclear cell layer; and
 - b) separating fetal nucleated cells present in the mononuclear cell layer from other cells present in the mononuclear cell layer on the basis of an antigen present on fetal nucleated cells, present on other cells present in the mononuclear cell layer, but not on both.
- 45 9. The method of Claim 8 wherein the sample of blood obtained from the pregnant woman is separated into constituent layers in step (a) by density gradient centrifugation and the fetal nucleated cells are fetal nucleated erythrocytes.

10. A method of separating fetal nucleated erythrocytes present in the blood of a pregnant woman, comprising enriching the proportion of fetal nucleated erythrocytes present in a sample of peripheral blood obtained from the pregnant woman to produce a maternal sample enriched in fetal nucleated erythrocytes, and sorting fetal nucleated erythrocytes present in the maternal sample enriched in fetal nucleated erythrocytes on the basis of an immunologic feature which is characteristic of fetal nucleated erythrocytes or characteristic of cells other than fetal nucleated erythrocytes, but not characteristic of both.

11. The method of Claim 10 wherein the proportion of fetal nucleated erythrocytes present in the sample of peripheral blood is enriched by separating the sample by density gradient centrifugation into constituent layers and removing the mononuclear cell layer.

12. A method of separating fetal nucleated erythrocytes present in a maternal blood sample, comprising the steps of:

- 15 a) separating the maternal blood sample into constituent layers, said constituent layers including a mononuclear cell layer;
- b) separating the mononuclear cell layer from the constituent layers produced in step (a);
- c) contacting the mononuclear cell layer with 1) a first monoclonal antibody, which is specific for an antigen present on fetal nucleated erythrocytes but not for maternal leucocytes and 2) a second monoclonal antibody, which is specific for an antigen present on maternal leucocytes but not on fetal nucleated erythrocytes, under conditions appropriate for binding of monoclonal antibody to specific antigen, thereby producing fetal nucleated erythrocyte/first monoclonal antibody complexes and maternal leucocyte/second monoclonal antibody complexes; and
- d) separating fetal nucleated erythrocyte/first monoclonal antibody complexes from maternal leucocyte/second monoclonal antibody complexes.

25 13. The method of Claim 12 wherein the first monoclonal antibody and the second monoclonal antibody are fluorescently labelled, each with a different fluorescent material and separation in step (d) is by flow cytometry.

30 14. A method of separating fetal nucleated erythrocytes present in a sample of blood obtained from a pregnant woman, comprising the steps of:

- a) initially enriching the proportion of fetal nucleated erythrocytes present in the sample of blood by separating non-nucleated erythrocytes from nucleated erythrocytes present in the sample on the basis of size and density, to produce an enriched maternal sample;
- 35 b) further enriching the proportion of fetal nucleated erythrocytes present in the enriched maternal sample by combining the enriched maternal sample with at least one detectable monoclonal antibody selective for fetal nucleated erythrocytes, under conditions appropriate for binding of detectable monoclonal antibody with fetal nucleated erythrocytes, to produce bound detectable monoclonal antibody; and
- c) separating bound detectable monoclonal antibody from the sample.

40 15. The method of Claim 14 further comprising,

- a) in step (b), combining the enriched maternal sample with at least two detectable monoclonal antibodies, wherein the first of said detectable monoclonal antibodies is selective for antigens present on mature human leucocytes and for antigens present on very immature erythrocyte precursors, but not for mature nucleated erythrocytes; the second of said detectable monoclonal antibodies is selective for an antigen which is present on the surface of fetal nucleated erythrocytes, but not for antigens present on mature human leucocytes or antigens present on very immature erythrocytes; and the two detectable monoclonal antibodies are separately detectable, to produce a first bound detectable monoclonal antibody and a second bound detectable monoclonal antibody; and
- b) in step (c), separating the first bound detectable monoclonal antibody and the second bound detectable monoclonal antibody from one another; and for example wherein the first of said detectable monoclonal antibodies is selected from the group consisting of HLe-1, L4 and M3 HLe-1 and the second of said detectable monoclonal antibodies is anti-TfR antibody.

55 16. A method of detecting the occurrence of fetal DNA of interest in fetal DNA in a sample of blood obtained from a pregnant woman, comprising the steps of:

- a) separating fetal nucleated cells present in the sample of peripheral blood from the sample, to produce sep-

- arated fetal nucleated cells;
- b) treating separated fetal nucleated cells to render DNA present in said cells available for hybridization with a complementary nucleotide sequence;
- c) contacting the product of step (b) with a selected DNA probe which is a nucleotide sequence complementary to the fetal DNA of interest, under conditions appropriate for hybridization of complementary DNA sequences to occur; and
- d) detecting hybridization between the product of step (b) and the selected DNA probe, the occurrence of hybridization being indicative of the presence of fetal DNA of interest in the fetal DNA.

5 10 17. A method of detecting the occurrence of a fetal DNA of interest in fetal DNA in a sample of peripheral blood obtained from a pregnant woman, comprising the steps of:

- a) separating fetal nucleated erythrocytes present in the sample of peripheral blood from the sample;
- b) amplifying DNA present in the separated fetal nucleated erythrocytes, to produce amplified fetal DNA;
- 15 c) treating amplified fetal DNA to render it available for hybridization with a complementary nucleotide sequence;
- d) combining the product of step (c) with a DNA probe which is a nucleotide sequence complementary to the fetal DNA of interest; and
- 20 e) detecting hybridization between the product of step (c) and the DNA probe, the occurrence of hybridization being indicative of the presence of fetal DNA of interest in the fetal DNA.

18. A kit for detecting fetal DNA of interest in a sample of maternal blood comprising:

- 25 a) at least one antibody which is selective for a surface antigen characteristic of fetal nucleated cells but not selective for a surface antigen characteristic of maternal leucocytes;
- b) selected DNA primers; and
- c) at least one DNA probe complementary to the fetal DNA of interest.

19. A kit for detecting fetal DNA of interest in a sample of maternal blood comprising:

- 30 a) at least one antibody which is selective for a surface antigen characteristic of fetal nucleated cells but not selective for a surface antigen characteristic of maternal leucocytes;
- b) a solid support having affixed thereon antibodies selective for the antibody of (a);
- c) selected DNA primers; and
- 35 d) at least one DNA probe complementary to the selected DNA.

20. The kit of Claim 19, wherein the solid support is a magnetic bead and further Comprising a magnet.

21. A kit for separating fetal nucleated cells present in a sample of blood obtained from a pregnant woman comprising:

- 40 a) at least one antibody which is selective for a surface antigen characteristic of fetal nucleated cells but not selective for a surface antigen characteristic of maternal cells; and
- b) a solid support having affixed thereon antibodies selective for the antibody of (a).

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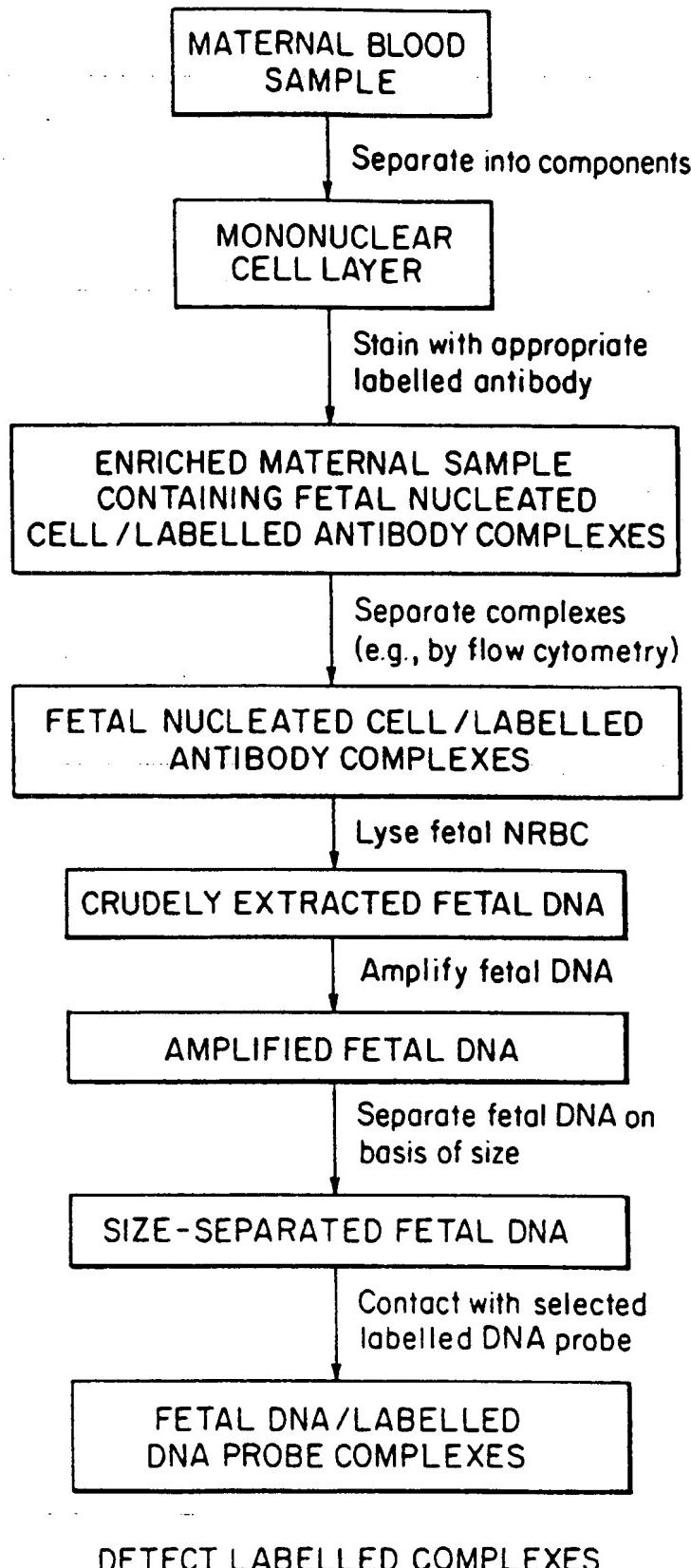
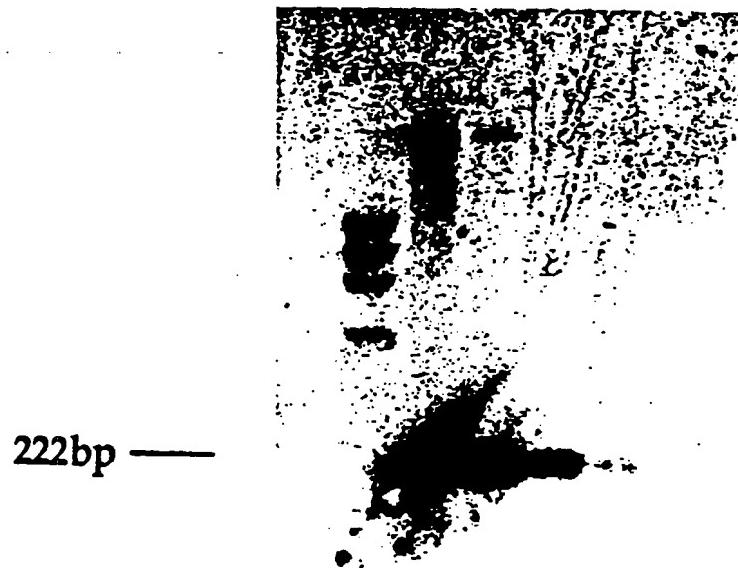


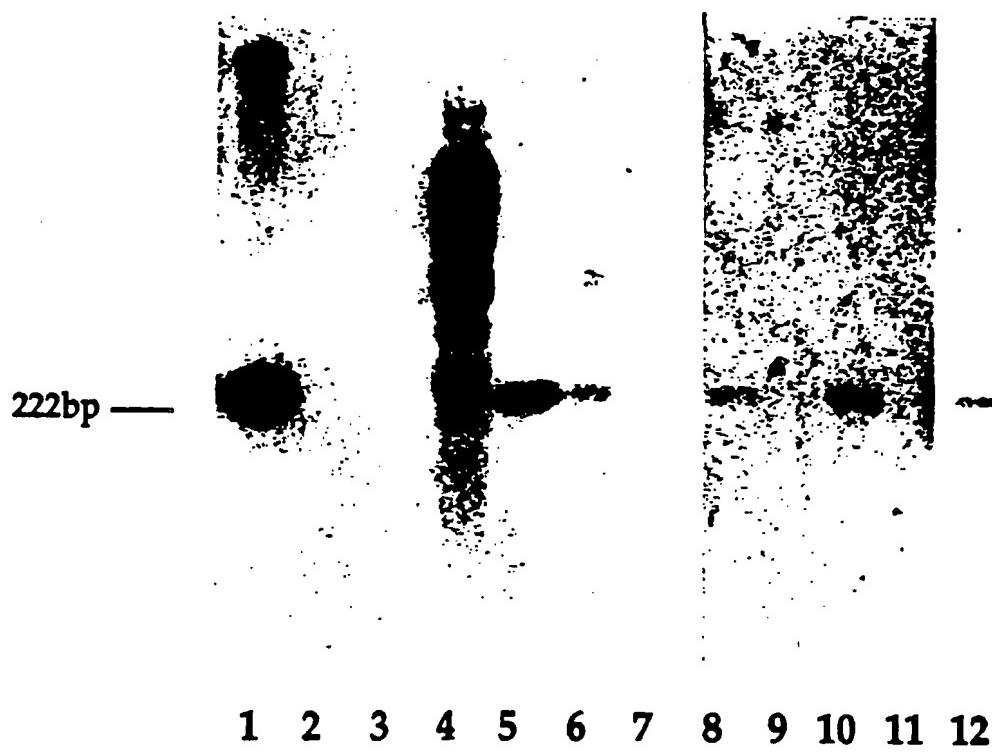
Fig. 1

FIG.2



1 2 3 4 5 6 7 8

FIG.3



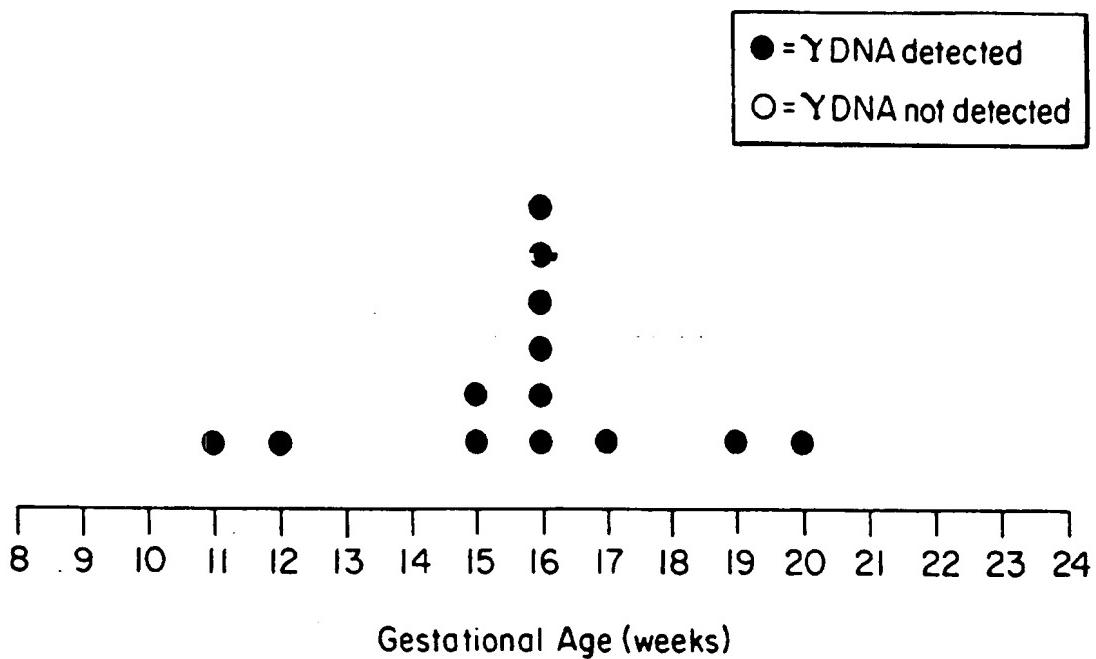


Fig. 4

FIG.5A

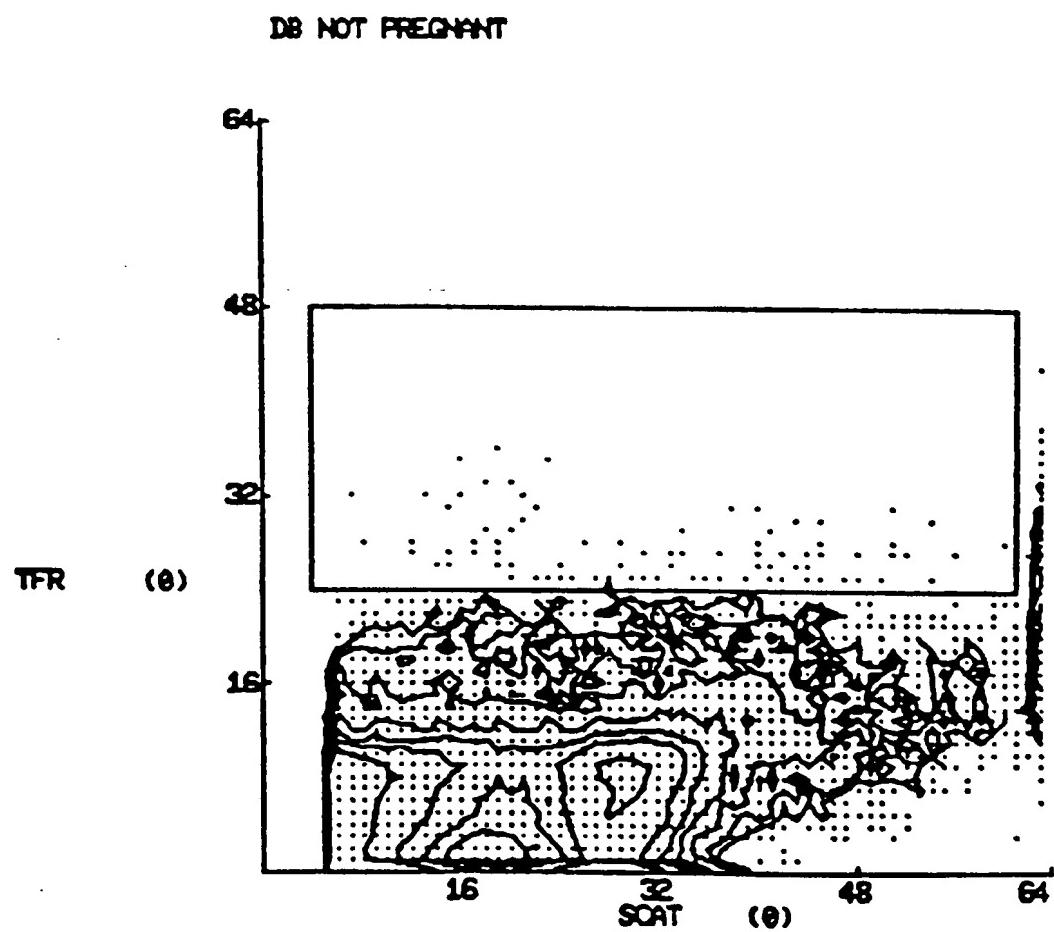


FIG.5B

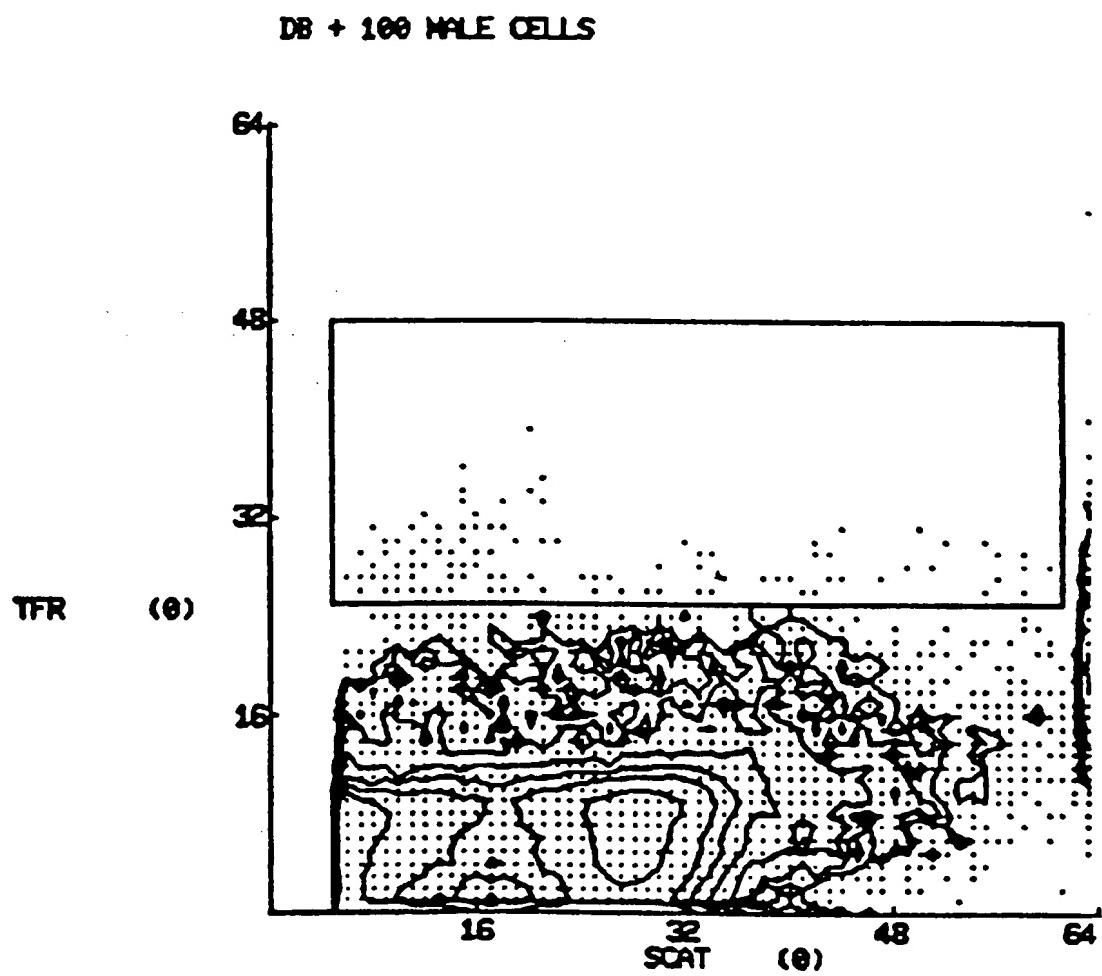


FIG.5C

DB+1000 MALE CELLS

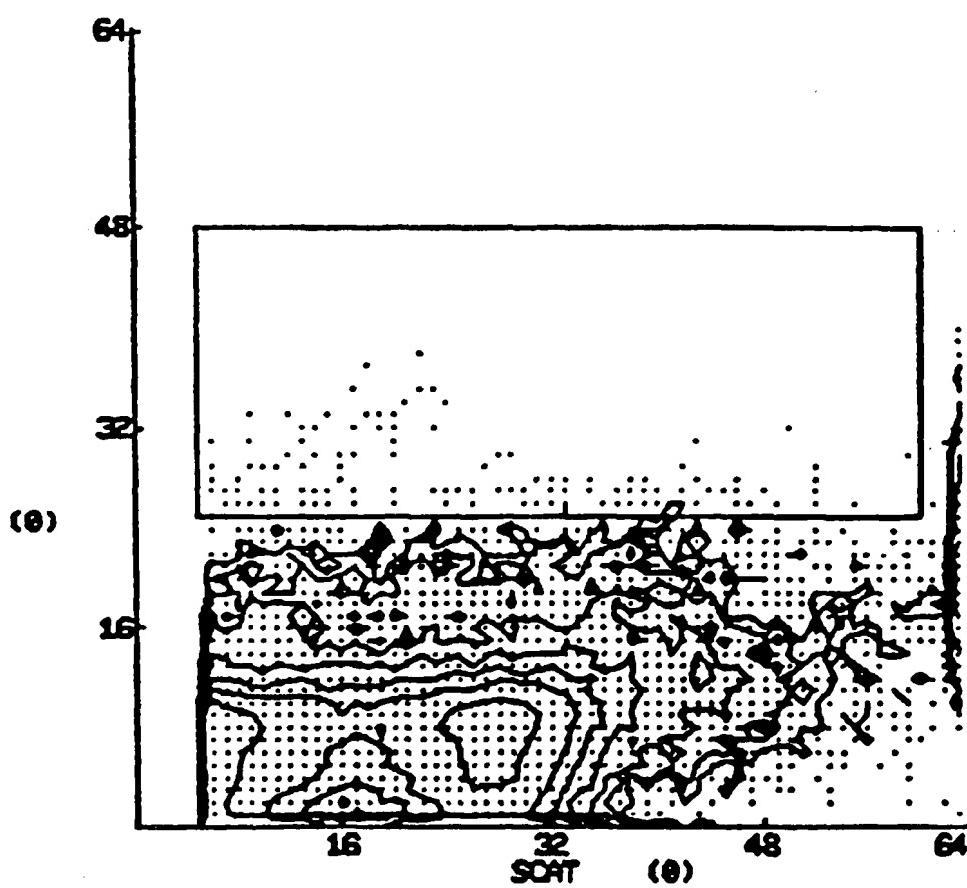


FIG.5D

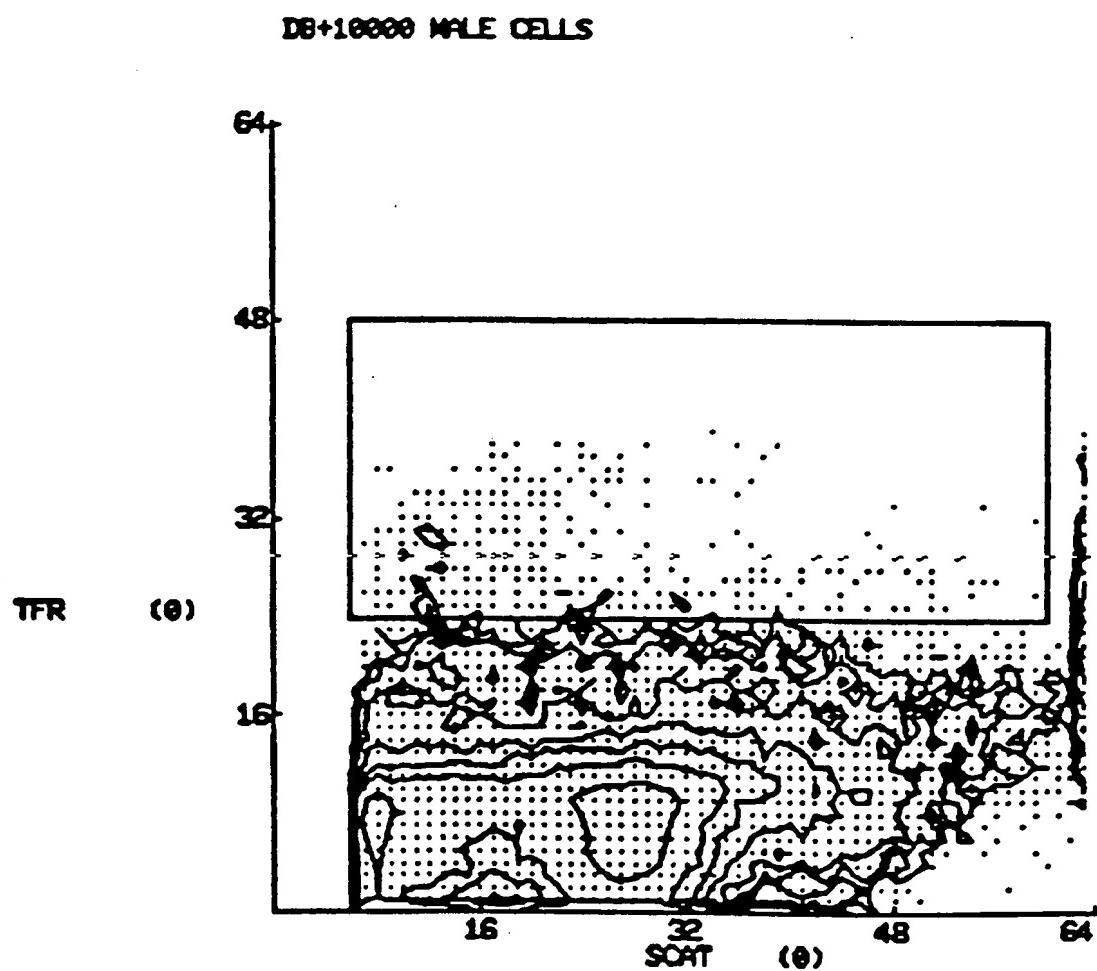


FIG.5E

DB+100,000 MALE CELLS

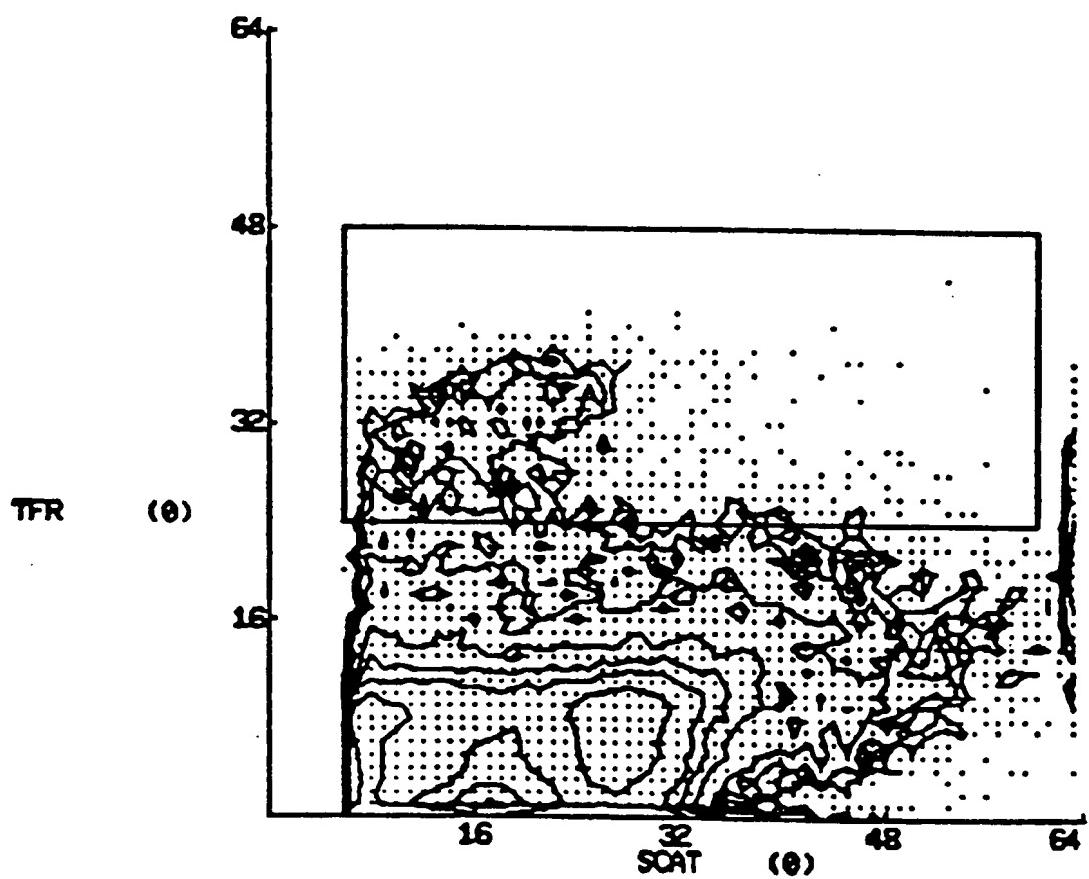


FIG.5F

DB+1,000,000 MALE CELLS

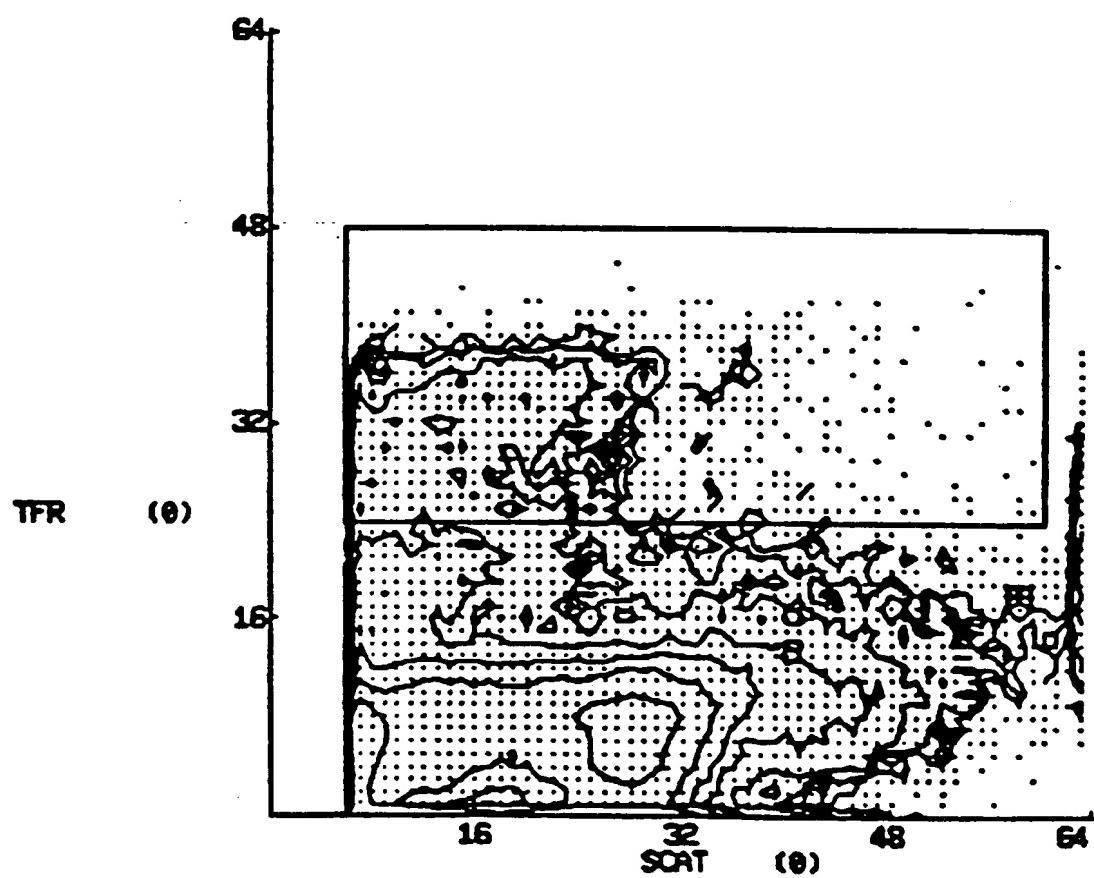
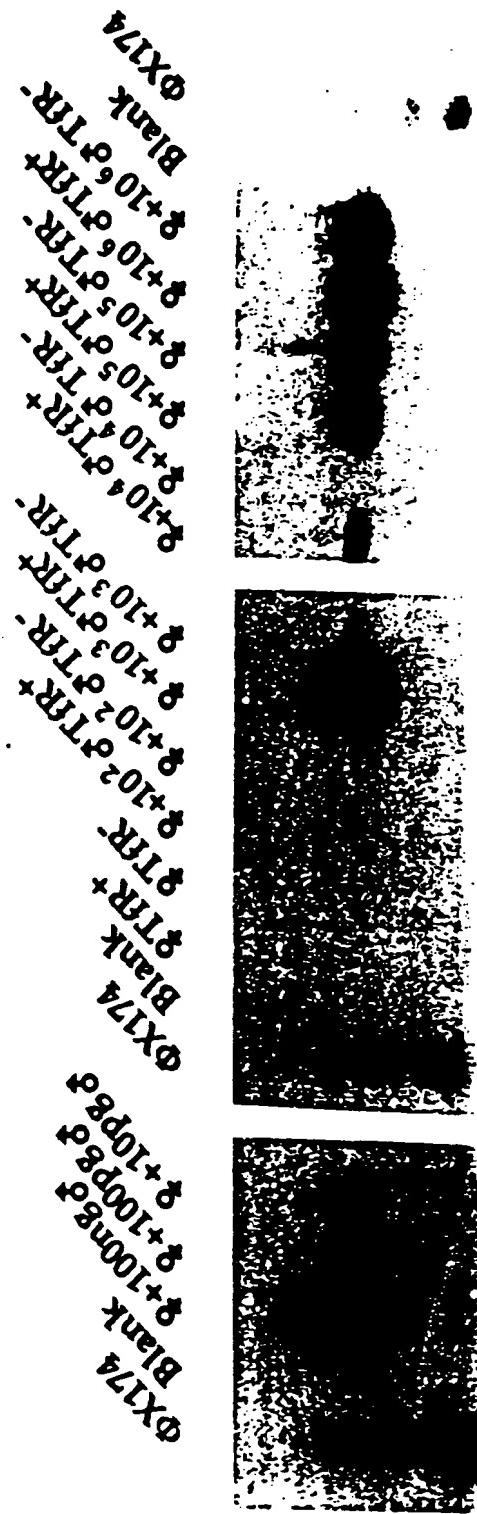


FIG. 6

Detection of Cord Blood Male DNA in
Reconstruction Experiments
(TfR antibody)



397bp —

FIG. 7A

MALE CORD BLOOD, POSITIVE CONT

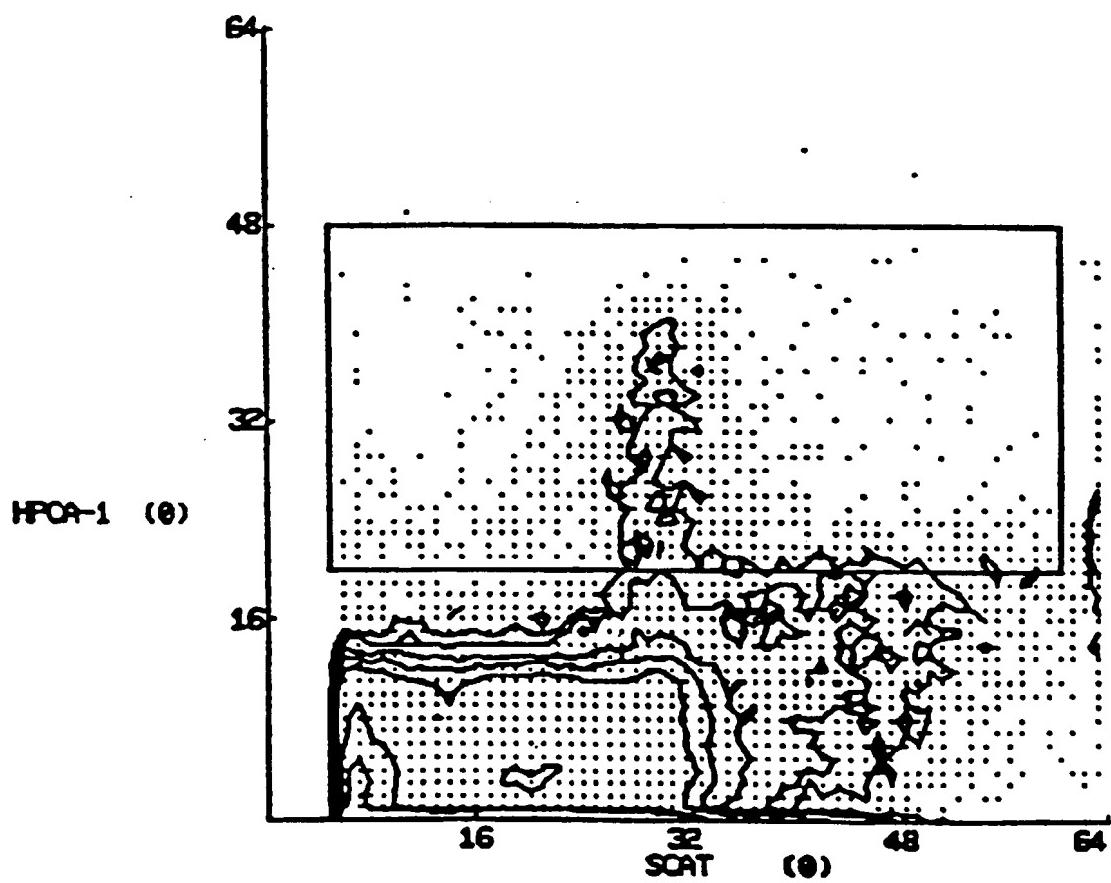


FIG.7B

MALE CORD BLOOD, NEG CONTROL

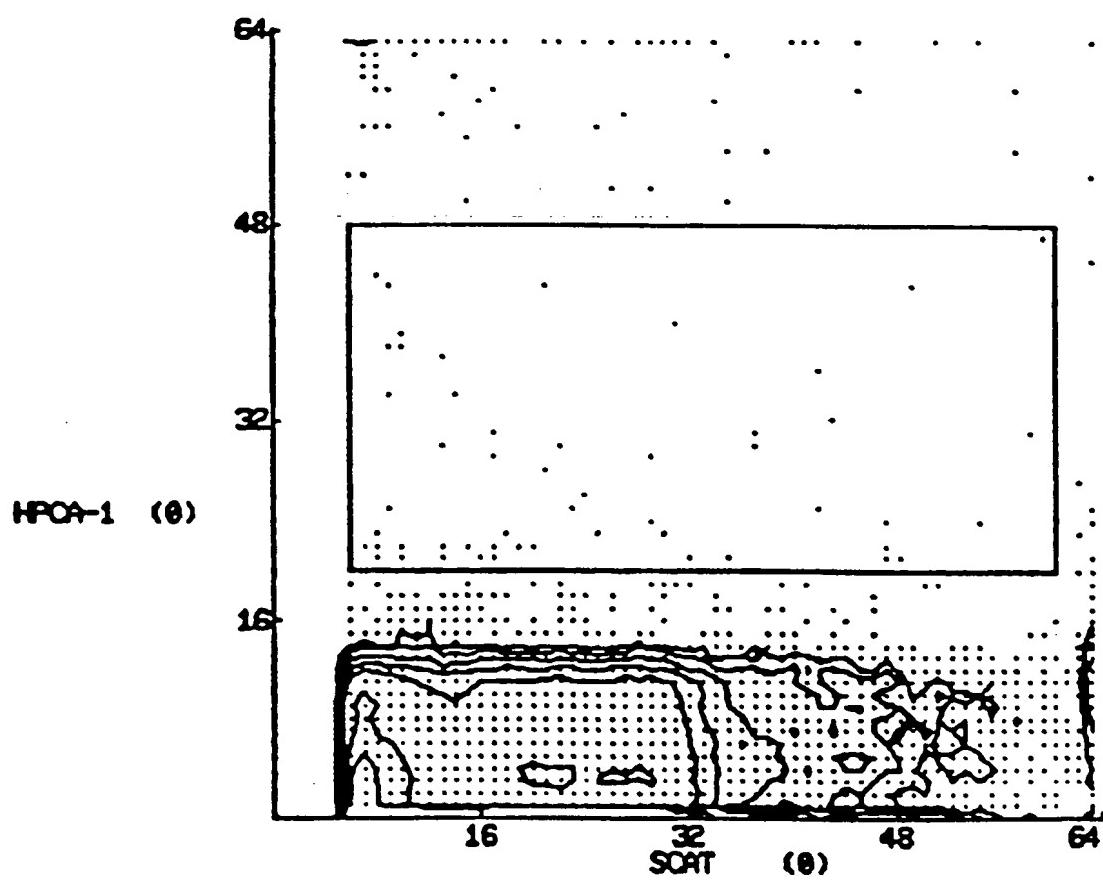


FIG. 7C

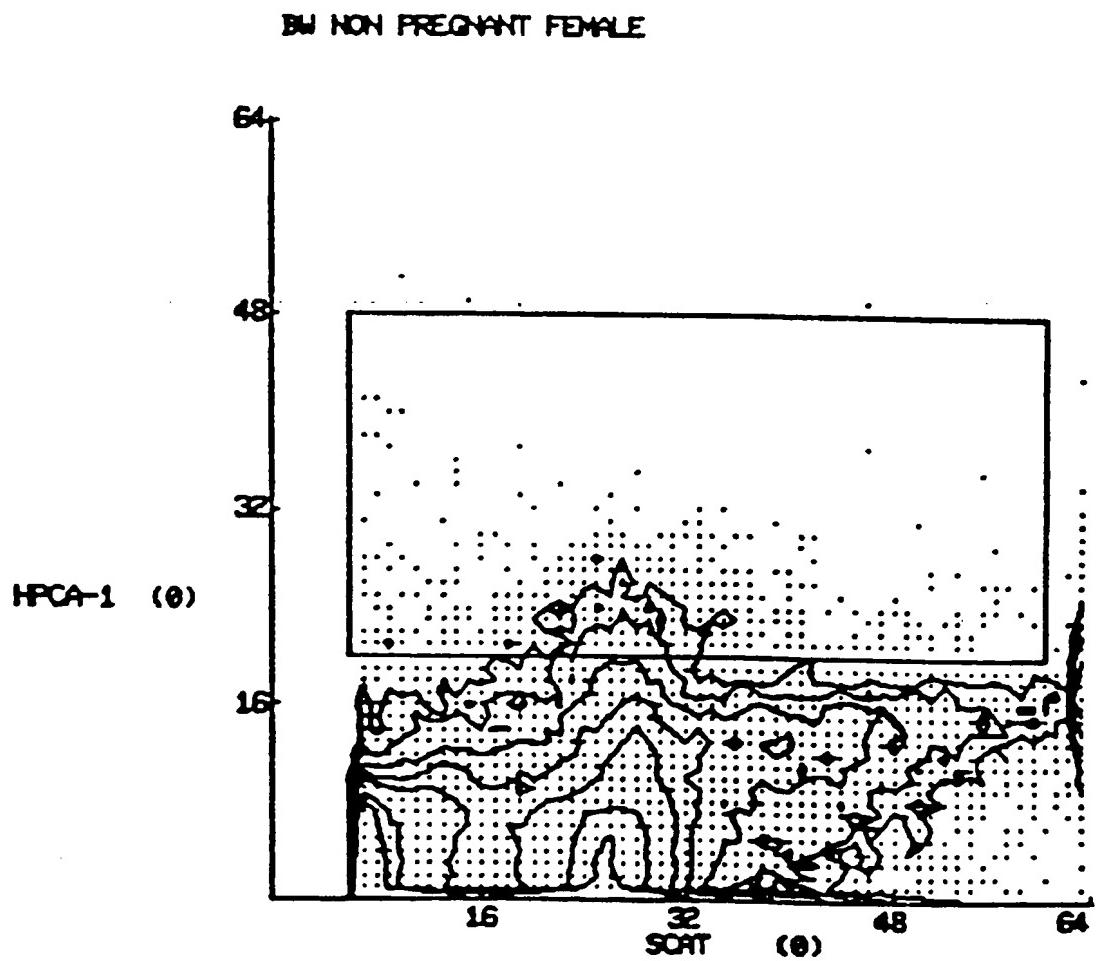


FIG. 7D

Bu+100 MALE CELLS

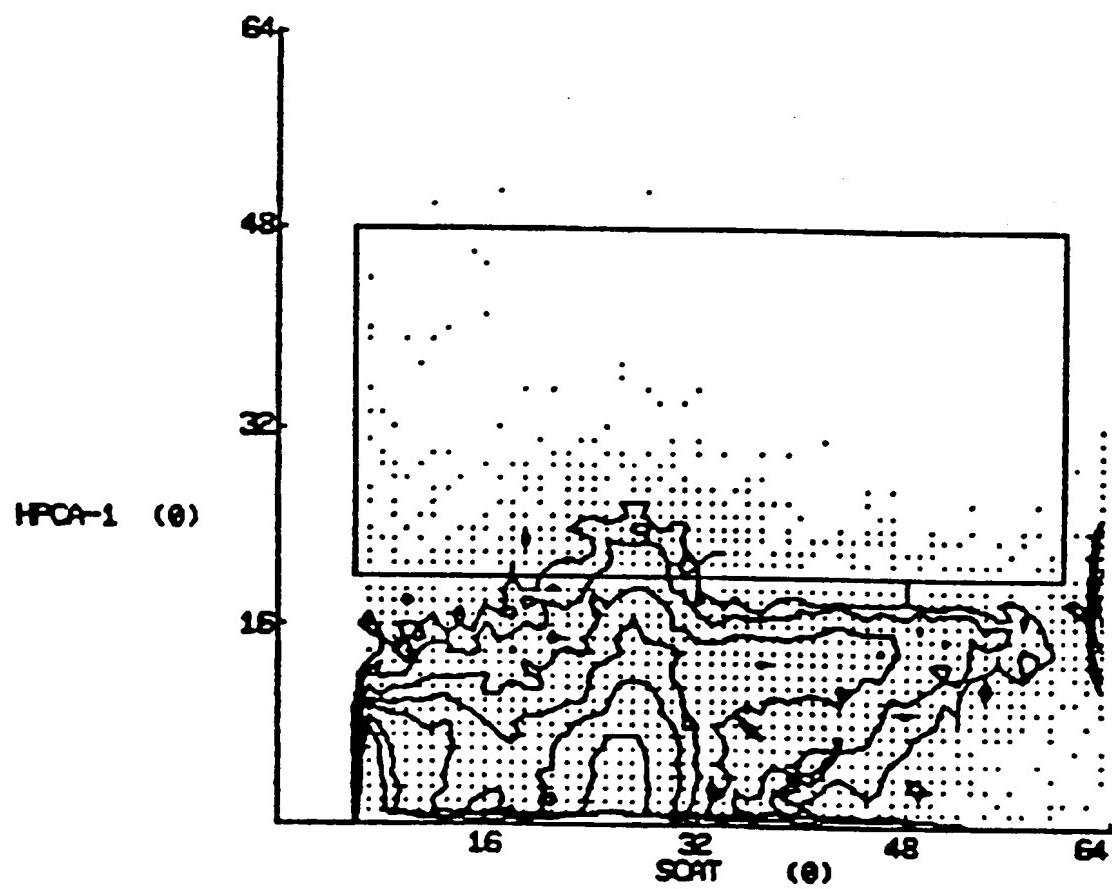


FIG.7E

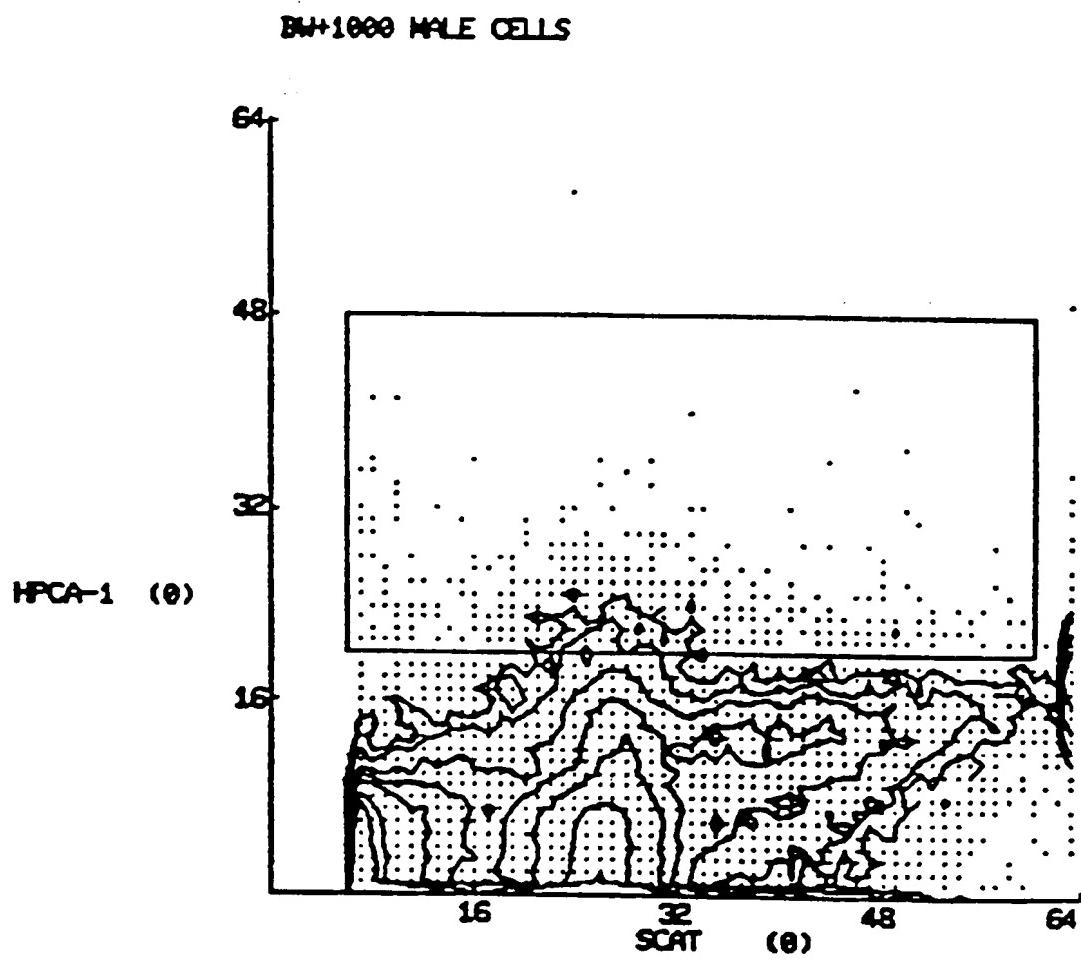


FIG.7F

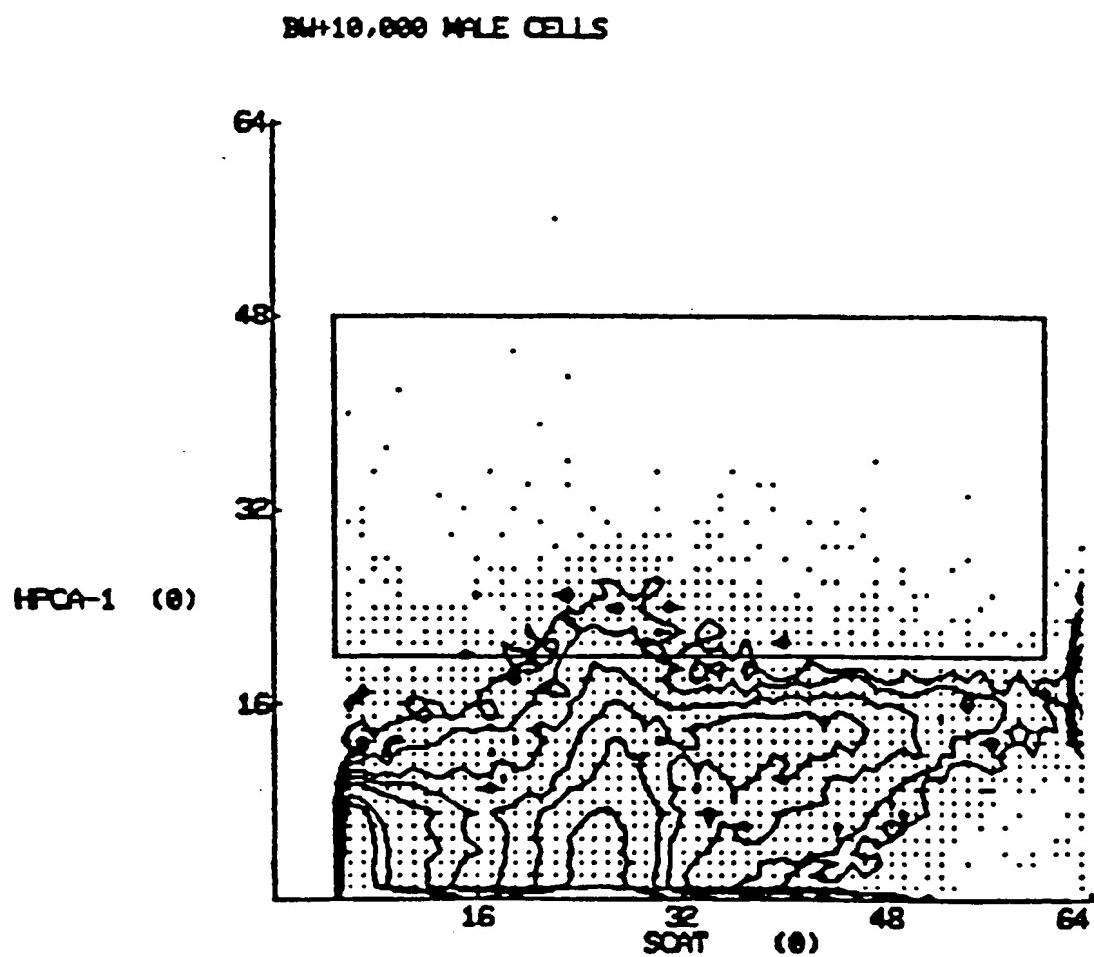


FIG.7G

BW+100,000 MALE CELLS

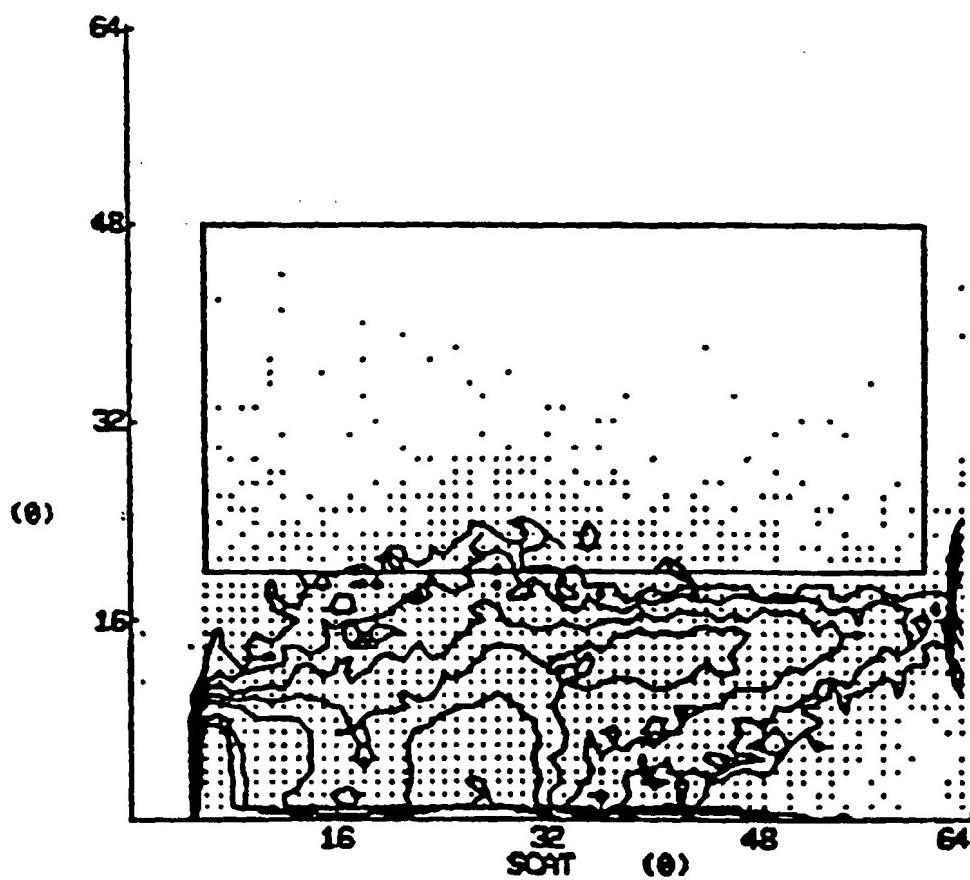


FIG. 7H

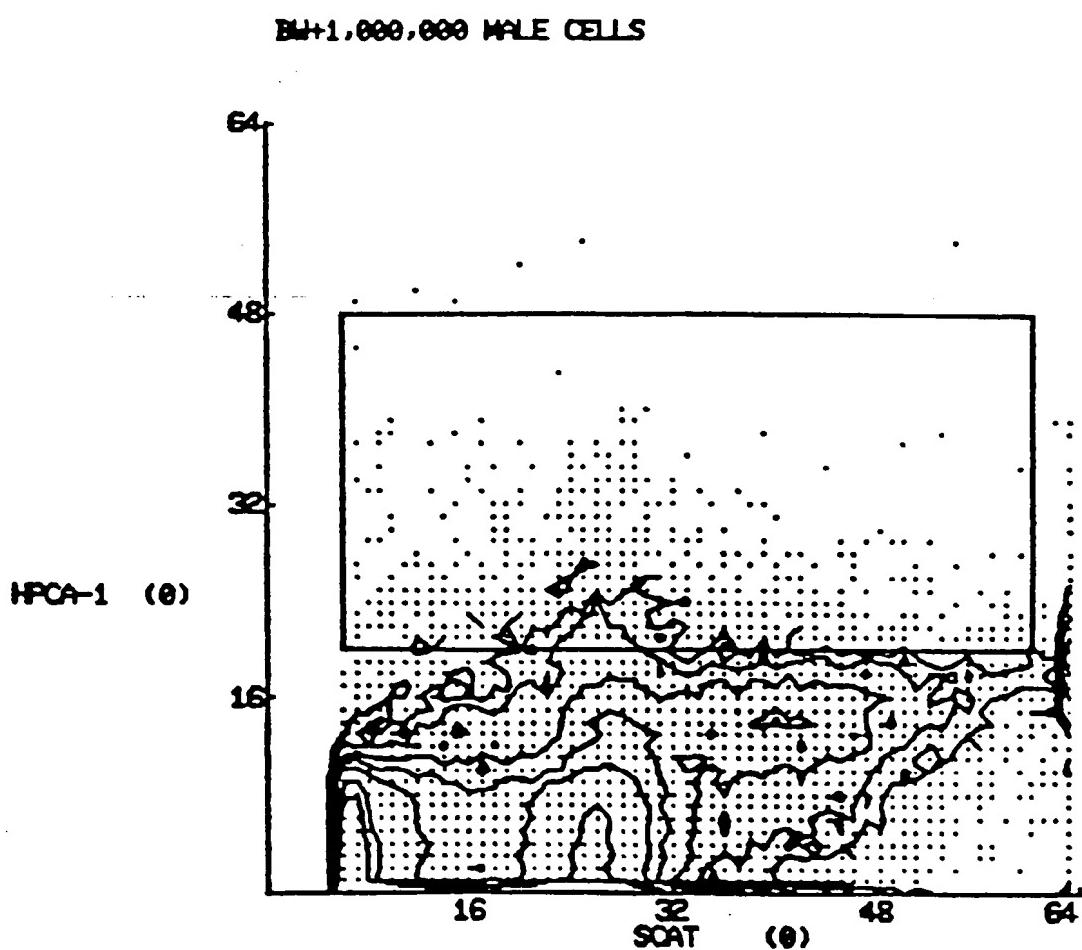
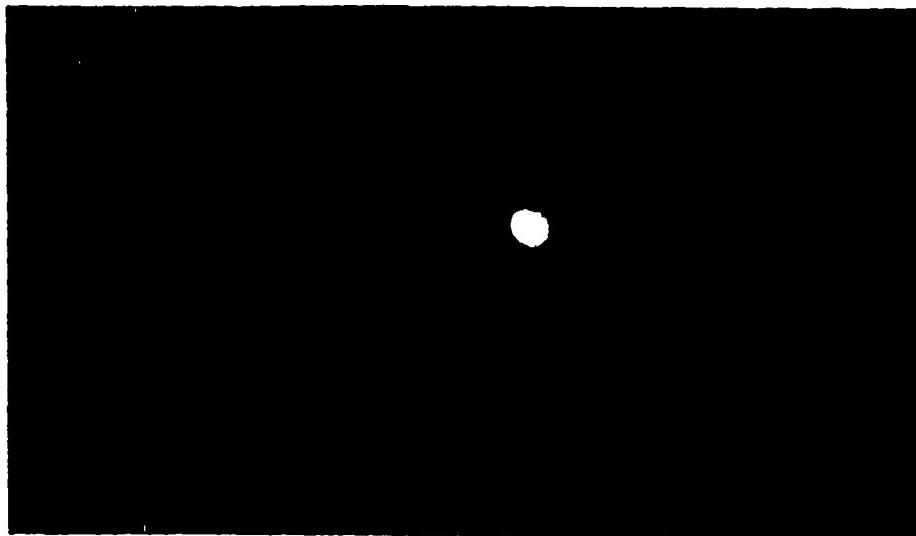


FIG.8





(19)

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Office européen des brevets



(11) EP 0 919 812 B1

(12)

EUROPEAN PATENT SPECIFICATION

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B01L 3/14, G01N 33/49,

G01N 33/569

(21) Application number: 98122238.3

(22) Date of filing: 23.11.1998

(54) Method for the detection, identification, enumeration and confirmation of circulating cancer cells and/or hematologic progenitor cells in whole blood

Verfahren zum Nachweis und zur Identifizierung, Zählung und Bestätigung von zirkulierenden Krebszellen und/oder hämatologischen Vorläuferzellen in Vollblut

Methode de détection, identification, énumération et confirmation de cellules cancéreuses et/ou de cellules souches hématologiques circulantes dans un échantillon de sang

(84) Designated Contracting States:

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WO-A-96/39185

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US-A- 5 403 714

US-A- 5 506 145

US-A- 5 593 848

US-A- 5 635 362

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- CHANG S K ET AL: "DETECTION OF CELL SURFACE ANTIGENS ON BIOPSYED HUMAN TUMOR CELLS USING MONOCLONAL ANTIBODY-CONTAINING FLUORESCENT MICROSPHERES." J CLIN LAB ANAL, (1987) 1 (4), 326-331., XP000869696

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Description**Technical Field**

[0001] This invention relates to a method and assembly for the detection, identification, enumeration and confirmation of circulating cancer and/or hematologic progenitor cells in an anticoagulated whole blood sample which is contained in a transparent sampling tube assembly. The detection, identification, enumeration and confirmation steps can all be performed *in situ* in the sampling tube assembly. More particularly, the method of this invention involves the centrifugal density-based separation of the contents of the blood sample in a manner which will ensure that any circulating cancer and/or hematologic progenitor cells in the blood sample are physically displaced by their density into a predetermined axial location in the blood sample and in the sampling tube assembly, and also into a restricted optical plane in the sampling tube assembly which is adjacent to the wall of the sampling tube, and finally into a very well-defined zone of that optical plane.

Background Art

[0002] Cytology is the science and technology involved in the morphological characterization of mammalian cells. Cytology has clinical utility in both human and veterinary medicine. Cytology is most often used to diagnose the presence or absence of malignancy in exfoliated or harvested cells: a) that are shed into a body cavity such as the pleural space or peritoneum; b) that are shed into a body fluid that is excreted as, for example, sputum or urine; c) that are obtained by scraping or brushing a body surface, such as the uterine cervix, the uterine cavity, or bronchial mucosa; or d) that are obtained by direct needle-mediated aspiration from a tumor such as tumors of the thyroid, breast, lung, or the like. The exfoliated or harvested cells are then typically fixed, stained and visually studied, usually by bright field microscopy, and then, if needed, by immunologic stains and/or other molecular techniques.

[0003] This year approximately five hundred sixty thousand people will die from solid tumors (predominantly carcinomas) in the USA. Many of these deaths could be prevented by early diagnosis of these malignancies. Unfortunately, with the possible exception of the Prostate Specific Antigen (PSA) test for prostate cancer, there is no practical and routine methods that have been found to be effective for early detection of solid tumors through blood analysis.

[0004] Through early detection of cervical cancer, the Pap smear has decreased mortality from cervical cancer in the United States by over seventy percent. Development of an analogous test for other solid tumors could have a similar impact on overall cancer mortality.

[0005] The presence of circulating cancer cells that are spontaneously shed by cancerous tumors into the

circulating blood stream which is supplying the tumors with oxygen and nutrients has been confirmed. The presence of such cells in the blood stream has been inferred for decades because of the spread of cancerous tumors by what has been described as the hematogenous route and on very rare occasions have been visualized in blood specimens. Recently sophisticated procedures which employ reverse transcriptase in conjunction with Polymerase Chain Reaction (PCR) have been able to detect the presence of tumor cells by their molecular signature in a significant number of patients with cancer, both when the cancer is localized and after it has spread.

[0006] An additional means of detecting circulating cancer cell employs a technology known as Fluorescent Activated Cell Sorting (FACS), such as that manufactured by Becton Dickinson and Company of Franklin Lakes, New Jersey. The FACS detection of circulating cancer cells involves detection of cancer cells by detecting fluorescent labeled antibodies which are directed against and bound to one or more epitopes that are present on or in cancer cells, and are not present on or in normal blood cells, and/or by detecting combinations of epitopes that are present on or in circulating normal blood cells and that may or may not be present on or in cancerous cells, or combinations of the aforesaid methods.

[0007] The FACS technology is thus based on cell highlighting, i.e., it is photometric and utilizes antibody-epitope specificity, and it cannot be used to morphologically analyze cells *in situ* in the FACS instrument. Both the reverse transcriptase/PCR, (the molecular method), and the FACS, (the immuno-phenotypic method), require that the origin of the tumor being sought be known in order to select for the specific molecular species or immuno-phenotypic signals. The aforesaid techniques have contributed to confirmation of the theory that cancer cells do circulate in the blood stream, but these techniques are not practical especially in point of care applications, by virtue of their cost and/or nature, for detecting the presence or absence of circulating tumorous cancer cells in the blood stream. Thus, there is no general or generic blood analyzing procedure for the detection and confirmation of the malignant nature of circulating cancer cells, regardless of their source, in a patient. In addition, neither the aforesaid molecular nor the immuno-phenotypic methods utilize *in situ*, i.e., in a closed sampling system, cytopathologically-based analyses to determine the morphometric characteristics of circulating cells which permit cancer cells to be identified and confirmed.

[0008] Since approximately eighty two percent of all cancers are epithelial in origin (seventy two percent of which are fatal), epithelial cancer cells should be detectable in circulating blood. While the presence of epithelial cells in the circulating blood stream does not, by itself, prove malignancy, it does alert the cytologist to the greater likelihood of malignancy since epithelial cells are

not normally seen in the circulating blood stream. In certain cases, however; such as after surgery; or as a result of physical trauma; or as a result of dental flossing, or in cases of prostatitis, for example, it is possible that non-malignant epithelial cells may be found in the circulating blood stream. Visual morphological analysis of cells is currently the most reliable way to distinguish cancerous epithelial cells from benign epithelial cells which are found in the circulating blood sample. One problem which exists in connection with attempts to detect circulating cancer cells in blood via morphological analysis relates to the fact that circulating cancer cells in blood are often virtually indistinguishable from circulating hematologic progenitor cells, or blasts, by cytological analysis alone.

[0009] The paucity of cancer cells that may be present in a sample of circulating blood would require the cytopathologist to carefully examine approximately ten million nucleated blood cells in order to find one cancer cell, and that one cancer cell would be randomly located in the ten million nucleated blood cells, which in turn will themselves be homogeneously dispersed in a sea of five billion non-nucleated cellular blood constituents, i.e., the erythrocytes, plus two hundred fifty million platelets, all of which will be found in one milliliter of blood. Such a task would be very time consuming, and is thus impractical for use in analyzing a patient's blood for the presence or absence of cancer cells.

[0010] Another type of rare circulating nucleated cells which may be found in a blood sample are hematologic progenitor cells (HPC's), which include blasts, stem cells, and other progenitors of normally circulating cells are not usually present in a sample of circulating blood at levels which can be detected by the use of presently available hematopoietic instruments, such as impedance counters and examination of stained peripheral blood. In patients who are receiving chemotherapy and in patients who are receiving human granulocyte colony-stimulating factor (HG-CSF), and other similar cytokines, HPC's are more likely to be present, but generally at very low numbers, i.e., at about one to one thousand per ml, or less, of the sample. Thus, low concentrations of HPC's in a blood sample renders the HPC's non-detectable by routine methods.

[0011] It is important to detect and enumerate the HPC's because their enumeration can permit more efficient harvesting of the HPC's for clinically important stem cell transplant therapies. Similarly, the detection of circulating cancer cells in patients whose HPC's are being harvested is important so that reinfusion into the patient of harvested circulating cancer cells can be minimized.

[0012] A technique has been developed to measure constituent layers in a complex material mixture by centrifuging a sample of the material mixture in a capillary or other tube which contains an insert, typically a float. The float is preferably cylindrical, and has a specific gravity which causes it to settle into the centrifuged mix-

ture to a degree which creates an annular free volume in the tube into which the layer, or layers to be measured will settle. The layers to be measured are thus physically elongated, and can thus be more easily and accurately measured. The aforesaid technique is described in U.S. Patents Nos. 4,027,660, issued June 7, 1977; 4,082,085 issued April 4, 1978; 4,156,570 issued May 29, 1979; and others. This technology is presently being marketed by Becton Dickinson and Company under the registered trademark "QBC". This "QBC" technology has been adapted for use in the isolation and identification of microfilarial infestation of a blood sample, as set forth in U.S. Patent No. 4,190,328, issued February 26, 1980. U.S. Patents Nos. 5,403,714, issued April 4, 1995; 5,496,704, issued March 5, 1996; 5,506,145, issued April 9, 1996; and others describe the use of the aforesaid "QBC" technology to assay anticoagulated whole blood for various analytes; and also to assay tissue samples for the presence or absence of cancerous tumor cells, wherein tissue samples are admixed with a saline buffer solution prior to analysis.

[0013] It is evident that there exists a compelling need for a simple procedure and a system for performing such a procedure whereby a sample of capillary blood or venous blood could be quickly and accurately analyzed for the presence or absence of circulating cancer cells and/or hematologic progenitor cells. Additionally, the procedure should enable one to differentiate cancer cells from hematologic progenitor cells; and also enable one to confirm the nature of detected cells, all *in situ*, in the blood sampling paraphernalia.

Disclosure of the Invention

[0014] This invention relates to a method and apparatus for visually or photometrically detecting circulating cancer and/or hematologic progenitor cells in an anticoagulated whole blood sample, which blood sample is contained in a transparent sampling tube. The detection and confirmation of circulating cancer and/or hematologic progenitor cells in the blood sample can be attained in a matter of minutes by utilizing the fact that circulating cancer cells which are of epithelial origin, and hematologic progenitor cells, when present in the circulating blood stream, have a different density than the other nucleated constituents of blood and, when gravimetrically separated, the epithelial cancer and/or hematologic progenitor cells will layer out in, or adjacent to, the platelet layer of the centrifuged blood sample. We have determined that circulating epithelial cancer and/or hematologic progenitor cells do not layer out by sedimentation, i.e., by size, in the centrifuged blood sample, but rather layer out by density in the centrifuged blood sample. The platelet layer is a blood constituent layer which is generally devoid of nucleated cells, and is also free of materials which are susceptible to DNA staining, thus allowing quick identification of nucleated cells which are in the vicinity of the platelet layer.

[0015] This invention allows *in situ*, i.e., in the sampling paraphernalia, visual morphometric analysis and also labeled epitopic analysis and identification of suspicious, i.e., large cells of low specific gravity, nucleated cells which are found in the centrifuged blood sample. The invention also can allow *in situ* analysis of the suspicious cells in the sampling tube assembly. Such analysis can confirm whether individual suspicious cells are epithelial and malignant; epithelial and benign; or non-epithelial in origin but are hematologic progenitor cells, all without removing the blood sample from the sampling tube. A significant advantage to using the "QBC" paraphernalia to isolate and identify circulating cancer and/or hematologic progenitor cells in anticoagulated whole blood is that the "QBC" paraphernalia provides a closed system which is not susceptible to cross contamination from other samples. This advantage is very important in a reliable rare event detection system.

[0016] The cancer and/or hematologic progenitor cells in question are found to be in the vicinity of the platelet layer in a blood sample which has been centrifuged in the aforesaid "QBC" tube and insert paraphernalia, when the blood sample is examined under appropriate magnification. The procedure of this invention thus involves two steps which are each performed *in situ* while the blood sample remains in the sampling tube.

[0017] One step involves the detection of characteristic epitopic highlighting on the cells to determine the epithelial origin, or hematologic progenitor origin, of the nucleated cells noted in the tube. This step can be characterized as an "epitopic" analysis. In performing the epitopic step, one can use epithelial-specific antigens, such as E-cadherin, Cadherin 11, Epithelial membrane antigen (EMA), Carcino embryonic antigen (CEA), Integrins, EP-CAM, MUC3, CD-44, growth factor receptors, such as epidermal growth factor (EGF) receptor, Hepatocyte growth factor (HGF) receptor, among others for detection of cells of epithelial origin.

[0018] In order to detect cells of HPC origin, HPC epitopic-specific labeled antibodies which are directed against CD-33, CD34, for example, may be used. Epithelial cells will not be recognized by the aforesaid HPC-specific antibodies, nor will the HPC cells be recognized by the labeled epithelial-specific antibodies or other binding particles. Liposome encapsulation of the label may be used to enhance the ratio of signal to noise, and/or to change the density of the targeted cells. Encapsulation of dyes in liposomes, modification of the liposomes with binding agents, and attachment of labeled liposomes to target analytes in a sample, are all described in U.S. Patent No. 5,593,848, issued January 14, 1997 to R. A. Levine et al.

[0019] The other step involves morphological examination of cells either to identify suspicious cells, or to confirm the malignant nature of the cells. The other step can therefore be characterized as a "morphometric" or "morphological" analysis. A universal morphometric stain such as acridine orange, DAPI, Hoechst, or

"SYTO" brand dyes, or the like can be employed in this step. These two steps can be performed in either order, i.e., either one can be used to identify suspicious cells in the blood sample, and the other can be used to confirm the malignant or benign nature of any suspicious cells. The decision to rely on either the epitopic or morphometric analysis, or both, to determine the malignancy of a cell is dependent upon the type of tumor(s) that encountered. In some cases, the morphometric features alone are sufficiently characteristic so as not to require any additional confirmatory test. In other cases, an epitopic analysis may alone be sufficient. It is generally desirable and prudent to use both the epitopic and morphometric analyses in assaying the blood sample.

[0020] Morphometric analysis of suspicious nucleated cells that are detected in the vicinity of the platelet layer in the centrifuged blood sample can be accomplished by a cytopathologist visually, analyzing the blood sample either *in situ* in the tube, or by the cytopathologist visually analyzing an image, or a series of images, of the suspicious cells, which images are captured *in situ* in the tube, either manually by a technician-operated camera, or are captured automatically by an automated imaging instrument. The visual and image

analysis or capture steps of the blood analysis method of this invention are all conducted with optical magnification of the centrifuged blood sample while the latter remains in the sampling tube. Morphometric analysis of captured images of nucleated cells from the blood sample can be remotely performed on the captured images.

[0021] Detection of nucleated cells which are suspected to be cancerous or of hematologic progenitor origin that are found in the centrifuged blood sample in the vicinity of the platelet layer can be based upon differential staining of the suspect cells as a result of the presence and/or absence of surface epitopes known to be present or absent on most epithelial cells, and/or on most epithelial cancer and/or hematologic progenitor cells, and are also known to be absent on normal circulating nucleated and non-nucleated blood cells or their precursors. Fluorophores or other detectable dyes or markers with distinctive emissions, such as Rhodamine, Fluorescein, Cy3, Cy5, Texas Red, Bodipy, or the like, can be coupled to antibodies or antigens, either directly, or after being encapsulated in liposomes as described in the aforesaid U.S. Patent No. 5,593,848.

[0022] Another way to detect nucleated cells in the blood sample involves the addition to the blood sample of a universal nucleated cell stain such as acridine orange, Hoescht, DAPI, or "SYTO" brand dyes for example, which are capable of differentially staining all nucleated cells that may be found in the blood sample so as to differentially highlight and clarify the morphology of all of the nucleated cells in the blood sample.

[0023] The epitopic stains and the universal stain will maximally fluoresce at different wavelengths, thus allowing the detection of suspicious cells visually or by means of an automated instrument. For example, the

centrifuged blood sample can be scanned by an appropriate instrument so as to identify all nucleated cells in the region of interest, and then scanned again with a different light filter set so as to identify all epithelial cells in the blood sample. In this way, cells can be identified which call for visual inspection for abnormal morphology. The visual morphometric examination can be performed as a preliminary detection test or it can be performed as the subsequent confirmatory test of the nature of the suspicious cells.

[0024] The preliminary morphometric visual analysis, or the photometric epitopic analysis, will be performed in the vicinity of the platelet layer of the expanded buffy coat in the blood sample. The fact that circulating cancer cells of epithelial origin, as exemplified by lung cancer, prostate cancer, breast cancer, rectal/colon cancer, ovarian cancer, and kidney cancer, among others, can be found in the vicinity of the platelet layer in a centrifuged sample of anticoagulated whole blood without the need of an extraneous density gradient, and can be morphologically and colorometrically identified *in situ* in the blood sample tube as being cancerous, is not described in the literature.

Furthermore, the fact that circulating hematologic progenitor cells, which are derived from the bone marrow and are precursors of leukemia, can be found without the need of an extraneous density gradient in the vicinity of the platelet layer in a centrifuged sample of anticoagulated whole blood and can be epitopically identified, is likewise not described in the literature.

[0025] It is therefore an object of this invention to provide a method and apparatus for detecting, identifying and confirming the presence or absence of circulating cancer and/or hematologic progenitor cells in a centrifuged anticoagulated whole blood sample which is contained in a transparent tube.

[0026] It is an additional object of this invention to provide a method and apparatus of the character described wherein the circulating cancer and/or hematologic progenitor cells are isolated from a vast majority of non-cancerous and non-hematologic progenitor nucleated blood cells in the blood sample.

[0027] It is a further object of this invention to provide a method and apparatus of the character described wherein the preliminary detection step may be performed either visually or epitopically by appropriate instrumentation, and also wherein the subsequent confirmation step can be performed either visually or epitopically.

[0028] It is a supplementary object of this invention to provide a method and apparatus of the character described wherein isolated nucleated cells in the centrifuged blood sample can be confirmed as malignant or benign (negative), or as hematologic progenitor cells, *in situ* in the tube.

[0029] It is a further object of this invention to provide a method and apparatus of the character described which enables enumeration of detected circulating can-

cer and/or hematologic progenitor cells in the blood sample.

[0030] It is another object of this invention to provide a method and apparatus of the character described wherein the blood sample analysis is performed *in situ* in a closed system which system is resistant to contamination from ambient surroundings, thereby reducing the possibility of false positive results.

[0031] These and other objects and advantages of the invention will become more readily apparent from the following detailed description of the invention when taken in conjunction with the accompanying drawings, in which:

15 Brief Description of the Drawings

[0032]

20 FIG. 1 is a side elevation view of a tube and float paraphernalia assembly which can be utilized to perform the procedure of this invention;

25 FIG. 2 is a schematic view of an automated microscopical instrument assembly which is adapted for use in conjunction with the paraphernalia of FIG. 1 to perform the procedure of this invention;

30 FIG. 3 is a graphic depiction of a photomicrograph taken of cultured breast cancer cells (MDA-MB-468) which were added to a sample of acridine orange-stained anticoagulated whole blood, and which cells were isolated, visually identified, and visually confirmed in the centrifuged blood sample using a 10X objective lens in an appropriately configured microscopical instrument assembly;

35 FIG. 4 is a graphic depiction of a photomicrograph taken of HT-29 colon cancer cells which were added to a sample of acridine orange-stained anticoagulated whole blood which cells were isolated, visually identified, and visually confirmed *in situ* in the tube containing the centrifuged blood sample using a 10X objective lens in an appropriately configured microscopical instrument assembly;

40 FIG. 5 is a graphic depiction of a photomicrograph taken of a single cultured HT-29 colon cancer cell added to a sample of acridine orange-stained anticoagulated whole blood using a 50X objective lens immersed in oil in an appropriately configured microscope assembly, which cell was isolated, visually identified, and visually confirmed *in situ* in the tube containing the centrifuged blood sample;

45 FIG. 6 is a graphic depiction similar to FIG. 5 of the photomicrograph taken *in situ* in a sampling tube of the single cultured HT-29 colon cancer cell in a sample of acridine orange-stained anticoagulated

whole blood using a 50X objective lens immersed in oil in an appropriately configured microscopical instrument assembly, wherein the cancer cell was highlighted by Cy3-labeled E-cadherin in the centrifuged blood sample:

FIG. 7 is a graphic depiction of a photomicrograph taken *in situ* in a sampling tube of cultured HT-29 colon cancer cells which were added to a sample of acridine orange-stained anticoagulated whole blood and which were isolated, visually detected and confirmed using a 200X objective lens immersed in oil in an appropriately configured microscopical instrument assembly;

FIG. 8 is a graphic depiction similar to FIG. 7 of the photomicrograph taken of the cultured HT-29 colon cancer cells which were added to a sample of acridine orange-stained anticoagulated whole blood and which were isolated, visually detected and confirmed using a 200X objective lens immersed in oil in an appropriately configured microscopical instrument assembly, wherein the cancer cells were highlighted by Cy3-labeled E-cadherin in the centrifuged blood sample;

FIG. 9 is a graphic depiction of a photomicrograph taken of cultured HT-29 colon cancer cells which were added to a sample of acridine orange-stained anticoagulated whole blood and which were isolated, visually identified at 200X magnification, and visually confirmed in the centrifuged blood sample;

FIG. 10 is a graphic depiction similar to FIG. 9 of the photomicrograph taken of cultured HT-29 colon cancer cells in the sample of acridine orange-stained anticoagulated whole blood at 200X magnification wherein the cancer cells were highlighted by Cy3-labeled E-cadherin in the centrifuged blood sample;

FIG. 11 is a graphic depiction of a photomicrograph taken of circulating breast cancer cells detected in a sample of acridine orange-stained anticoagulated whole blood taken from a patient known to have metastatic breast cancer, which cells were isolated, visually identified using a microscope assembly having a 50X objective lens, and visually confirmed *in situ* in the tube containing the centrifuged blood sample;

FIG. 12 is a graphic depiction similar to FIG. 11, but showing the circulating breast cancer cells highlighted by Cy3-labeled E-cadherin in the centrifuged blood sample;

FIG. 13 is a graphic depiction of a photomicrograph taken of circulating prostate cancer cells taken from

a patient known to have metastatic prostate cancer, and which cells were isolated, visually identified at 500X magnification, and visually confirmed *in situ* in the tube containing the centrifuged blood sample:

FIG. 14 is a graphic depiction similar to FIG. 13, but showing the circulating prostate cancer cells highlighted by Cy3-labeled E-cadherin in the centrifuged blood sample;

Detailed Description of the Invention:

[0033] Referring now to the drawings, there is shown in FIG. 1 a side elevational view of a sampling tube and float assembly, which is referred to hereinafter generally as "the paraphernalia" and which includes a transparent sampling tube 2 which contains an elongated plastic insert or float 4. The tube 2 has a lower end 6 which is closed off by means of a closure cap 10. The tube 2 can be a capillary tube, or it can be a larger tube such as is described in U.S. Patent No. 5,086,784, issued February 11, 1992. The thickness of the gap between the tube bore and the insert 4 will be at least about ten microns so as to be accessible to target cells.

[0034] FIG. 2 is a schematic depiction of an automated colorimetric microscopical instrument assembly, which is denoted generally by the numeral 12, and which can be used to scan a centrifuged blood sample that is contained in the paraphernalia shown in FIG. 1, and can, without human intervention, colorometrically differentiate between different types of cells in the layers being scanned, and can create and store or transmit an image of the cell layers being scanned. The instrument assembly 12 includes a stage 14 which includes at least one rotatable support 16 which engages the ends of the sample tube 2 and enables the sample tube 2 to be rotated about its axis as the contents of the tube 2 are scanned. A reversible electric motor 18 selectively rotates a drive screw 20 in opposite directions so that the tube 2 can be axially moved in one direction and then in the reverse direction as the tube 2 is rotated stepwise in the stage 14. In this manner, the entire circumference contents of the tube 2 can be scanned. The automatic embodiment of the instrument assembly 12 includes a CCD camera 22 which, by means of a beam splitter 24 and lens 26, is focused upon the annular sample-containing gap in the tube assembly 2, which gap is located between the tube bore wall and the outer surface of the insert 4. It will be appreciated that the operating range of the lens 26 will be at least equal to the thickness of the gap between the tube bore and the insert 4 in the tube 2. The CCD camera 22 views and records images of the sample through a plurality of different emission light wave filters 28, 30 and 32 which are mounted on a selectively rotatable filter wheel 34. The instrument assembly 12 also includes an excitation light source 35 which directs an excitation light beam at the sample tube 2 through the beam splitter 24 and the focusing lens 26.

A series of excitation light wave length filters 36, 38 and 40 are mounted on a selectively rotatable filter wheel 42. The excitation light beam is deflected by the beam splitter 24 toward the focusing lens 26, and is focused on the sample tube 2 by the lens 26. Thus, the two filter wheels 34 and 42 allow one to selectively control and vary the wave length of the excitation light source, as well as the emitted light source. A preprogrammed microprocessor controller 44 is operable to selectively control the rotation of the sample tube 2, the rotation of the filter wheels 34 and 42, and operation of the CCD camera 22. The controller 44 thus enables fully automatic operation of the instrument assembly 12 without the need of human intervention.

[0035] The instrument assembly 12 operates in the following manner to capture and record images of the results of scanning the blood sample contained in the tube 2 for suspicious nucleated cells, and also for confirming the malignant or benign nature of observed suspicious cells *in situ* in the blood sample. A venous or capillary sample of anticoagulated whole blood is drawn into the sampling tube 2 and insert 4 assembly. The blood sample will be admixed in the tube 2, or prior to being drawn into the tube 2, with a fluorescent morphological stain such as acridine orange, so that morphological characteristics of nucleated cells which are observed in the blood sample can be analyzed. The blood sample is also admixed with an epithelial cell-specific marker which is used to determine whether any suspicious cells noted in the blood sample are of epithelial origin. This confirmation procedure was chosen because all of the tumorous cancer cells which are being assayed are epithelial cells. A preferred antigen that is highly specific to a surface receptor on epithelial cells is E-cadherin. In order to tag any epithelial cells we prefer to use Cy3 conjugated directly to E-cadherin. The Cy3 is a marker that fluoresces at a different wavelength than acridine orange. The admixture of anticoagulated whole blood, acridine orange and E-cadherin/Cy3 is centrifuged for a time period of about five minutes in the sampling tube-insert assembly. The centrifuged sample is then placed in the supports 16 on the stage 14, and the instrument 12 is turned on. The CCD camera 22 will record images of the portion of the centrifuged blood sample as the latter is rotated and reciprocated back and forth through the focal plane of the camera 22. An image of the entire circumference of a target zone in the blood sample will thus be produced by the camera 22. Separate scans will be made, one of which will record the blood sample image as defined by an appropriate combination of the filters 28, 30, 32, 36, 38 and 40 which is selected so as to differentially fluoresce the acridine orange stain added to the sample. This scan will produce images of all nucleated cells in the zone of the blood sample being scanned. Another scan will record the blood sample image as defined by a second appropriate combination of the filters 28, 30, 32, 36, 38 and 40 which is selected so as to differentially fluoresce the

E-cadherin, Cy3 or other label. This scan will produce images of all of the nucleated cells in the scanned zone of the blood sample which are epithelial cells.

[0036] Additional filter combinations can be used for additional scans depending on what additional cellular information is being sought. Such additional useful information could include additional cancer cell-specific epitopes which will enable the cytopathologist to identify the origin of the cancer cells, i.e., whether they are prostate cancer cells, breast cancer cells, lung cancer cells, ovarian cancer cells, or the like, which epitopic information is presently available, or becomes known in the future. The aforesaid analysis of the blood sample can be made automatically by the instrument shown in FIG. 2, or it can be performed by visually scanning the sample. The scanning steps and the analysis of the results of the scanning steps can be performed in either order. Scanning of the acridine orange-highlighted cells allows one to identify all of the nucleated cells in the scanned zone, and also allows one to analyze the morphology of the nucleated cells in order to identify any cells which appear to have a morphology which suggests malignancy. Scanning of the E-cadherin/Cy3 highlighted cells allows one to identify which of the nucleated cells in the scanned zone are epithelial cells. Confirmation of the presence of an epithelial cell (E-cadherin/Cy3-highlighted) having abnormal cell morphology (acridine orange-highlighted) in the centrifuged blood sample alerts the cytopathologist to the strong likelihood of a cancerous tumor in the blood sample donor. A similar protocol can be employed to determine whether suspicious nucleated cell are hematologic progenitor cells.

[0037] Referring now to FIGS. 3-14, there are depicted the results of photometric imaging of scans of blood samples taken with the "QBC" paraphernalia, and using the aforesaid technology.

[0038] We conducted experiments wherein cultured cancerous tumor cells were added to blood samples, to test both the limits of tumor cell detection, as well as to verify the differential morphology, and to determine the location of the tumor cells in the gravimetrically formed blood constituent density gradient. These experiments confirmed the veracity of the above-described procedure for isolating, analyzing and confirming the presence of circulating tumorous cancer cells in anticoagulated whole blood samples.

[0039] FIGS. 3 and 4 show recorded images of the morphologic appearance of an acridine orange-stained cultured breast cancer cell line, MDA-MB-468, (FIG. 3) and an acridine orange-stained cultured colon cancer cell line, HT-29, (FIG. 4) which cultured cancer cell lines were added to respective 100 μ l samples of anticoagulated whole blood. The spiked blood samples were then analyzed in accordance with this invention. The blood sample analyses reliably and reproducibly identified the cultured breast and cultured colon cancer cells in the blood samples. The cells were generally seen in the platelet layer near the platelet-plasma interface. Visual

analysis of the highlighted cells made *in situ* in the sample confirmed that they were malignant.

[0040] FIG. 5 is a recorded image of a single, rather large acridine orange-stained HT-29 colon cancer cell which was isolated in a 100 μ l sample of blood that had been doped with a small concentration of cultured HT-29 cancer cells. The bright layer to the right of the cancer cell is an interface of the centrifuged platelet layer in the blood sample. This image was recorded at 500X magnification. Visual analysis of the highlighted cells made *in situ* in the sample confirmed that they were malignant.

[0041] FIG. 6 is a view similar to FIG. 5, but showing the isolated HT-29 colon cancer cell as it appears when viewed through the E-cadherin/Cy3 filter set. It will be noted that all other cells in the field are not highlighted, while the HT-29 colon cancer cell is clearly visible, thus confirming the fact that the large cell is an epithelial cell. Visual analysis of the highlighted cell made *in situ* in the sample confirmed that it was malignant.

[0042] FIG. 7 illustrates the recorded images of acridine orange-stained cultured HT-29 colon cancer cells taken at 200X magnification, when larger populations of the cultured cancer cells were added to the blood sample. With the larger population of colon cancer cells, the cancer cells were seen to be distributed more widely throughout the platelet layer and were concentrated in several locations, one at the lymphocyte-platelet interface, and another at the platelet-plasma interface. Visual analysis of the highlighted cells made *in situ* in the sample confirmed that they were malignant.

[0043] FIG. 8 is a view similar to FIG. 7 but showing the recorded images of E-cadherin/Cy3 stained colon cancer cells which confirms the epithelial origin of the highlighted cells. Visual analysis of the highlighted cells made *in situ* in the sample confirmed that they were malignant.

[0044] FIGS. 9 and 10 are illustrative of recorded images of acridine orange-stained cultured HT-29 colon cancer cells which were added to a blood sample, and which were taken at 10X magnification. The cancer cells were seen to be concentrated near the platelet-plasma interface. FIG. 9 shows the cancer cells morphologically highlighted by acridine orange; and FIG. 10 shows the cancer cells epitopically highlighted by E-cadherin/Cy3. Thus FIG. 9 confirms the presence of nucleated cells in the plasma layer adjacent to the platelet layer of the centrifuged blood sample; and FIG. 10 confirms that certain ones of the detected nucleated cells are epithelial cells. Visual analysis of the highlighted cells made *in situ* in the sample confirmed that they were malignant.

[0045] FIGS. 11 and 12 are illustrative of recorded images of acridine orange-stained circulating breast cancer cells in a blood sample taken from a patient known to be suffering from metastatic breast cancer. The cancer cells were seen to be concentrated near the platelet-plasma interface. FIG. 11 shows the cancer cells morphologically highlighted by acridine orange; and FIG. 12 shows the cancer cells epitopically highlighted by E-

cadherin/Cy3. Thus FIG. 11 confirms the presence of nucleated cells in the plasma layer adjacent to the platelet layer of the centrifuged blood sample; and FIG. 12 confirms that certain ones of the detected nucleated cells are epithelial cells. Visual analysis of the highlighted cells made *in situ* in the sample confirmed that they were malignant.

[0046] FIGS. 13 and 14 are illustrative of recorded images of acridine orange-stained circulating prostate cancer cells in a blood sample taken from a patient known to be suffering from prostate cancer. The cancer cells were seen to be concentrated near the platelet-plasma interface. FIG. 13 shows the cancer cells morphologically highlighted by acridine orange; and FIG. 14 shows the cancer cells epitopically highlighted by E-cadherin/Cy3. Thus, FIG. 13 confirms the presence of nucleated cells in the plasma layer adjacent to the platelet layer of the centrifuged blood sample; and FIG. 14 confirms that certain ones of the detected nucleated cells are epithelial cells. Visual analysis of the highlighted cells made *in situ* in the sample confirmed that they were malignant. The fact that not all cells are highlighted by Cy3 markers provides an internal negative control which confirms that the epitopically highlighted cells are epithelial in origin. Non-epitopically highlighted nucleated cells are lymphocytes.

[0047] Experiments were also conducted to determine the sensitivity of the aforesaid assay.

[0048] The standard "QBC" capillary tube holds 100 μ l of blood which contains 1×10^9 of red blood cells (RBCs) and 1×10^6 of nucleated cells (granulocytes, lymphocytes, etc.). Thus, without changing the scale of the test, the theoretical limit of sensitivity would be 1 cell in 1×10^6 of nucleated cells. Serial dilutions of HT-29 colon cancer cells were used to obtain multiple paired 10 μ l aliquots containing between 1 and 10 cells, or pairs containing between 10 and 100 cells. The first aliquot of the pair was added to the "QBC" tubes and the second was counted with a standard hemocytometer. These experiments led to the conclusion that the limit of sensitivity of this assay approaches the theoretical limit of 1 cell in 1×10^6 of nucleated cells using a 110 μ l tube. Theoretically the sensitivity of the test can be increased up to ten fold by performing the analysis in a 1ml blood sampling tube.

[0049] Although morphometric analysis may be sufficient for identification of cancer cells, other methods of verification may also be necessary. The assay of this invention takes advantage of the fact that it can detect abnormal cell morphology, and can also, at the same time, verify the epithelial or hematologic progenitor origin of any abnormal nucleated cells noted in the blood sample. Since the analysis of this invention is nondestructive of the cells, the cells may be removed from the sampling tube for additional analysis by other methods such as the PCR method described in the prior art, or by biochemical assay.

[0050] As an example we chose E-cadherin since this

antigen is highly specific for epithelial cells and is displayed on the external surface of the cell membrane. For these studies we used Cy3, which is a cyanamine-based fluorophore, and which was conjugated directly to E-cadherin monoclonal antibodies to be able to visualize cell staining at a wavelength other than that used for morphometric examination using acridine orange-induced fluorescence.

[0051] We have confirmed that malignant nucleated epithelial cells can be morphometrically identified in a centrifuged sample of anticoagulated whole blood using the technique of this invention. Suitable morphometric criteria which can be visualized in the blood sample *in situ* in the tube assembly include: intracellular nuclear/cytoplasmic ratios; intracellular nuclear size and shape; intracellular nuclear chromatin pattern; the thickness and size of the nuclear membrane; and the number and size of nucleoli; among other things. We have also determined that epithelial cancer cells and hematologic progenitor cells layer out in the centrifuged anticoagulated whole blood sample by density, rather than by sedimenting out in the blood sample by size. This determination allows the detection of circulating cancer cells and/or hematologic progenitor cells in a predetermined and known zone in the centrifuged blood sample, i.e., in the zone of the centrifuged blood sample where the platelets layer out. If the circulating cancer and/or hematologic progenitor cells were to sediment out in the blood sample by size, one would be unable to define an "zone of interest" where the cancer and/or hematologic progenitor cells would be expected to be found. The cancer and/or hematologic progenitor cells have been found predominantly near the platelet/plasma interface; within the platelet layer near the lymphocyte/platelet interface; or in the lymphocyte layer in artificially overloaded cases, all depending upon the concentration of cancer and/or hematologic progenitor cells which are in the blood sample. A theoretical sensitivity of the technique of this invention, when employing a 100 μ l capillary tube containing 1x10⁶ nucleated cells, is one detected cancer and/or hematologic progenitor cell in 1x10⁶ nucleated blood cells in a 100 μ l blood sample is attainable. As noted above, a ten fold increase in the theoretical sensitivity should be achievable if the volume of the blood sample were increased ten fold, to about one milliliter. Verification of the origin of cancer and/or hematologic progenitor cells in the blood sample can be confirmed by immunofluorescent labeling of suspicious cells. Thus visual inspection of the cells will determine whether they display cancerous morphometric characteristics, and immunofluorescence will verify the origin of the suspicious cells being inspected.

[0052] It will be appreciated that the aforesaid procedures and apparatus can be used to screen patients for the presence or absence of cancer cells; can be used to assess staging of a malignant tumor; can be used to assess the effectiveness of chemotherapy on patients being treated for cancer; and can be used to identify and

enumerate hematologic progenitor cells in the blood sample. The detection and enumeration of hematologic progenitor cells and cancer cells is of clinical importance for stem cell harvesting and purging of cancer cells from harvested stem cells. The use of this invention as a means to assess the effectiveness of chemotherapy provides a much more sensitive and rapid way to evaluate the therapy than does CAT scanning, X-ray, or the like which are presently used to monitor the size of a tumor. The effectiveness of chemotherapy may be assessed by counting the number of cancer cells in the blood sample. The counting procedure can be performed throughout the entire periphery of the well-defined zone of the tube, or it can be performed throughout only a portion of the periphery of the aforesaid zone of the tube. When the latter approach is taken, the number of cancer cells in the sample can be extrapolated by solving the formula:

$$C = N(360^\circ/d) + V;$$

wherein "C" is the resultant cell concentration; "N" is the number of target cells counted; "d" is the degree of rotation of the tube which was examined for target cells divided by "V" which is the volume of the sampling tube. The cell enumerating can be performed by means of a photometric counter, or can be done visually. The photometric approach can use a combination of epitopic labels which will differentially highlight either cancer and/or hematologic progenitor cells or other non-cancer cells. In this manner the highlighted and/or non-highlighted cells will be counted. The morphometric analysis can also be performed photometrically. The visual approach can use a morphometric stain such as acridine orange or the other morphometric stains identified above.

[0053] Advantages of the "QBC" technique and apparatus to diagnose and enumerate cancer cells in circulating blood over the FACS and molecular techniques include: 1) the relatively short period of time needed to perform the blood analysis; 2) the fact that the system can be integrated into standard laboratory equipment that all pathologists are capable of using without extensive training; 3) unfixed cells can be examined in a fluid medium so as to eliminate fixation artifacts; 4) only a relatively small blood volume is needed to perform the analysis; 5) the technique is equally sensitive as the molecular technique in that one cancer cell can be detected in a sample containing 10⁶-10⁷ normal nucleated cells; 6) the fact that the "QBC" technique utilizes a closed sampling and analysis system so as to eliminate cross contamination, which is a major problem in the molecular procedure; 7) the elimination of cellular contamination due to contaminating floating cells in fixation stains which are used in routine cytological procedures; and 8) the analysis of this invention is safer for the technicians performing the analysis since they will not be ex-

posed to the blood sample being analyzed.

[0054] The specific insert and tube shown in the drawings are cylindrical; however, they could also be made polygonal. The only limiting factor regarding the transverse configurations of the tube and insert is that they be complimentary with each other. The analysis of the blood sample is made under suitable magnification by a microscopical instrument, preferably equipped with a CCD camera. The gap formed in the tube between the tube and the insert is transversely sized so that individual target cells can be isolated and can be readily discerned, enumerated and morphometrically analyzed within the gap. The transverse thickness of the gap is also within the focal operating range of the microscopical instrument being used to analyze the gap.

[0055] It will be appreciated that the method of this invention, in its broadest sense, involves detecting the presence or absence of individual circulating target nucleated cells in a centrifuged sample of anticoagulated whole blood contained in a tube that also contains a generally cylindrical insert. The insert forms a well-defined annular zone in the tube. The blood sample is combined with one or more epitope-specific labeling agents that are operative to produce a characteristic signal result on target nucleated cells, which result can include no signal at all, and which result defines the presence or absence of one or more epitopes on the target nucleated cells. The blood sample is also combined with a colorant which is operable to clarify cell morphology in all nucleated cells in the blood sample. Circulating nucleated cells are thus identified by cell morphology, and all identified nucleated cells which by reason of their morphology may be target cells are further characterized as target or non-target cells epitopically. By way of further explanation, assume that a specific combination of epitopes "A" and "B" is characteristic of a target cell, but not characteristic of other cells in the blood sample. The presence or absence of only one of these epitopes; or the presence or absence of both of these epitopes could be characteristic of the target cell. Thus, any one of four different respective epitope-specific labeling agent signal results of: A and no B; B and no A; both A and B; or no A and no B, could be used to characterize the target cell. The identifying and characterizing steps can be performed *in situ* in the tube. Obviously, more, or less, than two different epitopes could be employed in the characterization of target cells.

[0056] Since many changes and variations of the disclosed embodiment of the invention may be made without departing from the inventive concept, it is not intended to limit the invention otherwise than as required by the appended claims.

Claims

1. A method for detecting cancer cells and/or hematologic progenitor cells in a sample of anticoagulated

whole blood, said method comprising the steps of:

a) providing a sample of anticoagulated whole blood containing an epitopic cell labeling agent operable to differentiate cancer cells and/or hematologic progenitor cells from other nucleated cells in the sample, said sample being contained in a transparent tube which also contains an insert creating a well-defined gap between the tube and the insert;

b) centrifuging the sample of blood in the tube so as to gravimetrically separate the blood sample into its constituent components by density;

c) the insert of step (a) having such a specific gravity which causes it to settle into the centrifuged sample to a degree that any nucleated cells to be detected will be layered out in said gap;

d) examining said gap in order to determine whether any epitopically differentiated cancer cells and/or hematologic progenitor cells are present in said gap.

2. The method of claim 1, wherein
the blood sample further contains a stain which is operable to clarify cell morphology in all nucleated cells in the blood sample,
and wherein the cell morphology of any nucleated cells present in the gap after centrifugation thereof is examined.

3. The method of claim 1 or 2, wherein
any cancer cells and/or hematologic progenitor cells which are found to be present in said gap are enumerated.

4. The method of any one of claims 1 to 3, wherein
the gap which is examined, is the zone wherein platelets in the blood sample have gravitated during centrifugation, or a zone adjacent thereto.

5. The method of any one of claims 1 to 4, wherein
the blood sample is combined with one or more epitope-specific labeling agents operable to differentiate by epitopically highlighting nucleated epithelial cells which may be present in the blood sample,
the blood sample is combined with a stain operable to clarify cell morphology in nucleated cells in the blood sample,
any nucleated epithelial cells found in said gap after centrifugation are enumerated *in situ*,
the cell morphology of any nucleated cells found in said gap after centrifugation is examined *in situ*,

- said combining steps being performed either before or after the blood sample is placed in the tube, and said enumerating and examining steps being performed in no particular order.
6. The method of any one of claims 1 to 4, wherein the blood sample is combined with one or more epitope-specific labeling agents operable to differentiate by epitopically highlighting hematologic progenitor cells which may be present in the blood sample,
- the blood sample is combined with a stain operable to clarify cell morphology in nucleated cells in the blood sample,
- any hematologic progenitor cells found in said gap after centrifugation are enumerated in situ,
- the cell morphology of any nucleated cells found in said gap after centrifugation is examined in situ,
- said combining steps being performed either before or after the blood sample is placed in the tube, and said enumerating and examining steps being performed in no particular order.
7. The method of any one of claims 3 to 6, wherein said enumerating and examining steps are performed with an automated microscopical instrument.
8. The method of claim 7, wherein gap has a transverse thickness which is essentially equal to a focal operating range of the microscopical instrument at a predetermined power.
9. The method of claim 8, wherein said transverse thickness is within a range of ten to one hundred microns.
10. The method of any one of claims 1 to 9, wherein a blood sampling tube is used which contains an axially elongated, generally cylindrical insert which forms an annular gap between the tube and the insert.
- Patentansprüche**
1. Verfahren zum Nachweis von Krebszellen und/oder hämatologischen Vorläuferzellen in einer Probe von antikoaguliertem Gesamtblut, aufweisend die folgenden Schritte:
- a) Zur Verfügung stellen einer Probe von antikoaguliertem Gesamtblut, welches ein epitopisches Zellenmarkierungsmittel enthält, das geeignet ist, Krebszellen und/oder hämatologische Vorläuferzellen von anderen kernhaltigen Zellen in der Probe zu unterscheiden, wobei die Probe in einer transparenten Röhre enthalten
- 5 ist, welche auch einen Einsatz enthält, der einen gut definierten Spalt zwischen der Röhre und dem Einsatz erzeugt;
- b) Zentrifugieren der Blutprobe in der Röhre, um gravimetrisch die Blutprobe in ihre konstituierenden Komponenten aufgrund der Dichte zu trennen;
- c) der Einsatz aus Schritt a) hat eine derartige spezifische Dichte, welche bewirkt, dass er bis zu einem Grad in der zentrifugierten Probe zum Ruhen kommt, dass irgendwelche zu detektierende kernhaltige Zellen in dem genannten Spalt geschichtet sind;
- d) Untersuchen des Spalts, um festzustellen, ob epitopisch differenzierte Krebszellen und/oder hämatologische Vorläuferzellen in dem Spalt vorhanden sind.
2. Verfahren nach Anspruch 1, bei welchem die Blutprobe außerdem einen Farbstoff enthält, welcher geeignet ist, die Zellmorphologie aller kernhaltigen Zellen in der Blutprobe zu verdeutlichen; und wobei die Zellmorphologie von irgendwelchen nach Zentrifugation in dem Spalt vorhandenen kernhaltigen Zellen untersucht wird.
3. Verfahren nach Anspruch 1 oder 2, wobei irgendwelche Krebszellen und/oder hämatologische Vorläuferzellen, welche in dem Spalt vorgefunden werden, ausgezählt werden.
4. Verfahren nach einem der Ansprüche 1 bis 3, wobei der Spalt, welche untersucht wird, jene Zone ist, bzw. eine dazu benachbarte Zone ist, in welche Plättchen in der Blutprobe während der Zentrifugation durch Gravitation gelangt sind.
5. Verfahren nach einem der Ansprüche 1 bis 4, bei welchem die Blutprobe mit einem oder mehreren Epitop-spezifischen Markierungsmitteln gemischt wird, welche (s) geeignet sind (ist), durch epitopisches Markieren kernhaltige Epithelzellen zu differenzieren, welche in der Blutprobe vorhanden sein können, die Blutprobe mit einem Farbstoff gemischt wird, welcher geeignet ist, die Zellmorphologie kernhaltiger Zellen in der Blutprobe zu verdeutlichen, irgendwelche kernhaltige Epithelzellen, welche in dem Spalt nach Zentrifugation gefunden werden, in situ ausgezählt werden, die Zellmorphologie irgendwelcher kernhaltiger Zellen, welche nach Zentrifugation in dem Spalt gefunden werden, in situ untersucht wird, wobei die Schritte des Mischens entweder vor oder nachdem die Blutprobe in der Röhre platziert wird,

- durchgeführt werden, und die Schritte des Auszählens und Untersuchens in keiner bestimmten Reihenfolge durchgeführt werden.
6. Verfahren nach einem der Ansprüche 1 bis 4, bei welchem die Blutprobe mit einem oder mehreren Epitop-spezifischen Markierungsmittel gemischt wird, welche (s) geeignet sind, durch epitopisches Markieren hämatologische Vorläuferzellen zu differenzieren, welche in der Blutprobe vorhanden sein können, die Blutprobe mit einem Farbstoff gemischt wird, welcher geeignet ist, die Zellmorphologie in kernhaltigen Zellen in der Blutprobe zu verdeutlichen, irgendwelche hämatologische Vorläuferzellen, welche in dem Spalt nach Zentrifugation gefunden werden, in situ ausgezählt werden, die Zellmorphologie irgendwelcher kernhaltiger Zellen, welche nach Zentrifugation in dem Spalt gefunden werden, in situ untersucht wird, die Schritte des Mischens entweder bevor oder nachdem die Blutprobe in der Röhre platziert wird, durchgeführt werden, und die Schritte des Auszählens und Untersuchens in keiner bestimmten Reihenfolge durchgeführt werden.
7. Verfahren nach einem der Ansprüche 3 bis 6, wobei die Schritte des Auszählens und Untersuchens mit einem automatisierten mikroskopischen Instrument durchgeführt werden.
8. Verfahren nach Anspruch 7, wobei der Spalt eine Dicke in Querrichtung hat, welche im wesentlichen gleich einem Fokusbetriebsbereich des mikroskopischen Instruments bei einer vorher bestimmten Vergrößerung ist.
9. Verfahren nach Anspruch 8, bei welchem die Dicke in Querrichtung in einem Bereich von 10 bis 100 µm ist.
10. Verfahren nach einem der Ansprüche 1 bis 9, wobei eine Blutprobenröhre verwendet wird, welche einen axial länglichen, im allgemeinen zylindrischen Einsatz enthält, welcher einen ringförmigen Spalt zwischen der Röhre und dem Einsatz bildet.
- Revendications**
1. Procédé de détection de cellules cancéreuses et/ou de cellules souches hématologiques dans un échantillon de sang complet anticoagulé, ledit procédé comprenant les étapes de :
- a) préparation d'un échantillon de sang complet anticoagulé, contenant un agent de marquage cellulaire épitopique, pouvant différencier des cellules cancéreuses et/ou cellules souches hématologiques, d'autres cellules nucléées dans l'échantillon, ledit échantillon étant présent dans un tube transparent, qui contient également un insert créant un intervalle bien défini entre le tube et l'insert ;
- b) centrifugation de l'échantillon de sang dans le tube de manière à séparer par gravimétrie l'échantillon sanguin en ses composants constituants par densité ;
- c) l'insert de l'étape (a) ayant une masse spécifique telle qu'il se place dans l'échantillon centrifugé à un niveau tel que n'importe quelle cellule nucléée à détecter sera rassemblée dans ledit intervalle ;
- d) examen dudit intervalle afin de déterminer si des cellules cancéreuses et/ou des cellules souches hématologiques différenciées de manière épitopique, quelconques, sont présentes dans ledit intervalle.
2. Procédé selon la revendication 1, dans lequel l'échantillon sanguin contient en outre, un colorant, qui peut fonctionner pour clarifier la morphologie cellulaire de toutes les cellules nucléées dans l'échantillon sanguin, et dans lequel la morphologie cellulaire de n'importe quelle cellule nucléée dans l'intervalle après centrifugation est examinée.
3. Procédé selon la revendication 1 ou 2, dans lequel n'importe quelle cellule cancéreuse et/ou cellule souche hématologique, que l'on trouve dans ledit intervalle, est énumérée.
4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel l'intervalle qui est examiné, est la zone dans laquelle les plaquettes de l'échantillon sanguin se sont rassemblées pendant la centrifugation, ou une zone adjacente à celle-ci.
5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel l'échantillon sanguin est combiné à un ou plusieurs agents de marquage spécifiques d'épitope, pouvant différencier par marquage épitopique, des cellules épithéliales nucléées, qui peuvent être présentes dans l'échantillon sanguin, l'échantillon sanguin est combiné à un colorant pouvant fonctionner pour clarifier la morphologie cellulaire des cellules nucléées dans l'échantillon sanguin, on énumère n'importe quelle cellule épithéliale nucléée trouvée dans ledit intervalle après centrifuga-

tion, *in situ*,
la morphologie cellulaire de n'importe quelle cellule
nucléée trouvée dans ledit intervalle après centrifu-
gation, est examinée *in situ*,
lesdites étapes de combiner étant réalisées avant 5
ou après que l'échantillon sanguin ait été placé
dans le tube, et
lesdites étapes d'énumération et d'examen n'étant
pas réalisées selon un ordre particulier.

10

6. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel l'échantillon sanguin est combiné à un ou plusieurs agents de marquage spécifiques d'épitope, pouvant différencier par marquage épito- 15
pique, des cellules souches hématologiques, qui peuvent être présentes dans l'échantillon sanguin,
l'échantillon sanguin est combiné à un colorant pou-
vant fonctionner pour clarifier la morphologie cellu-
laire des cellules nucléées dans l'échantillon san- 20
guin,
on énumère n'importe quelle cellule souche héma-
tologique trouvée dans ledit intervalle après centri-
fugation, *in situ*,
la morphologie cellulaire de n'importe quelle cellule 25
nucléée trouvée dans ledit intervalle après centrifu-
gation, est examinée *in situ*,
lesdites étapes de combiner étant réalisées avant
ou après que l'échantillon sanguin ait été placé
dans le tube, et
lesdites étapes d'énumération et d'examen n'étant 30
pas réalisées selon un ordre particulier.

25

7. Procédé selon l'une quelconque des revendications 3 à 6, dans lequel lesdites étapes d'énumération et 35
d'examen sont réalisées avec un instrument mi-
croscopique automatisé.

35

8. Procédé selon la revendication 7, dans lequel l'in- 40
tervalle a une épaisseur transversale qui est essen-
tiellement égale à une amplitude de fonctionnement
focal de l'instrument microscopique à une puissan-
ce prédéterminée.

40

9. Procédé selon la revendication 8, dans lequel ladite 45
épaisseur transversale se situe dans un intervalle
allant de 10 à 100 µm.

45

10. Procédé selon l'une quelconque des revendications 1 à 9, dans lequel un tube d'échantillonnage san- 50
guin est utilisé, lequel contient un insert axialement
allongé, généralement cylindrique, qui forme un in-
tervalle annulaire entre le tube et l'insert.

50

55

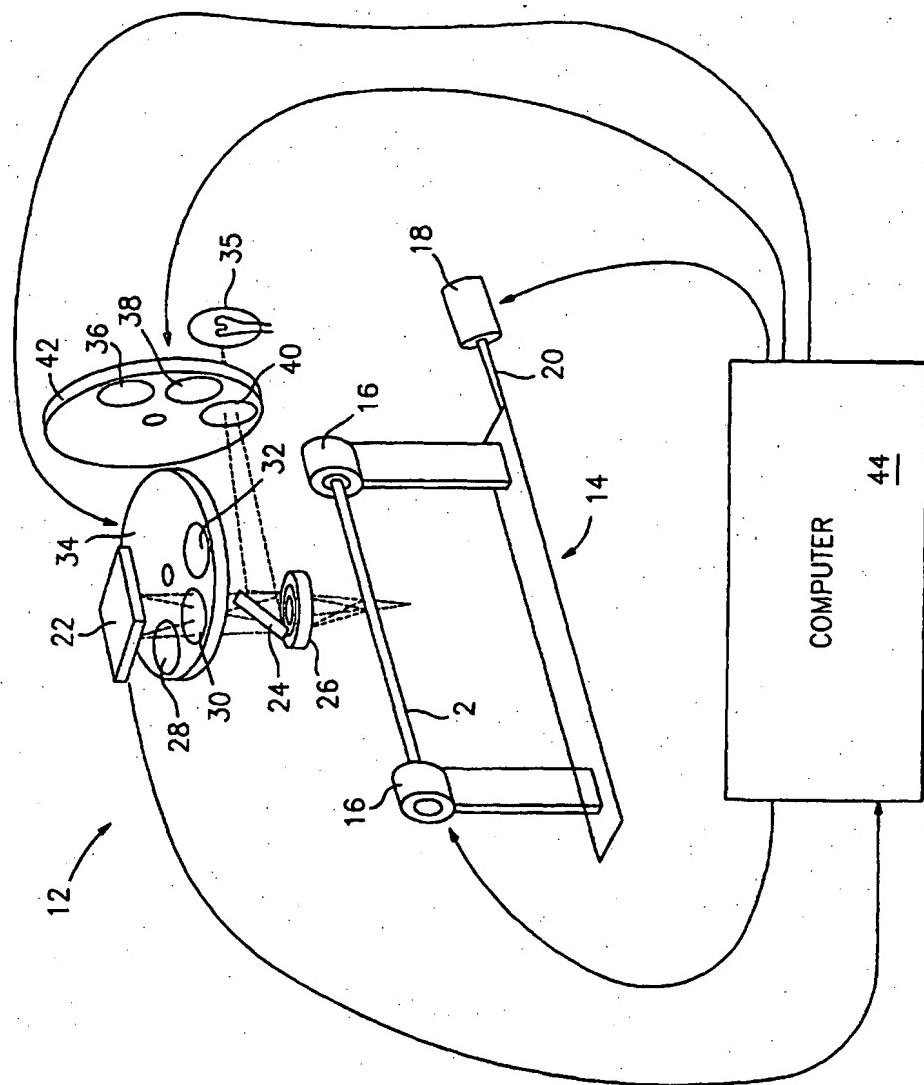


FIG. 2

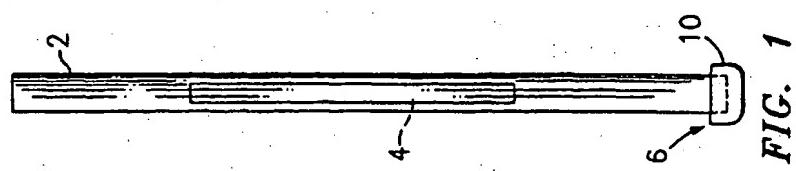


FIG. 1

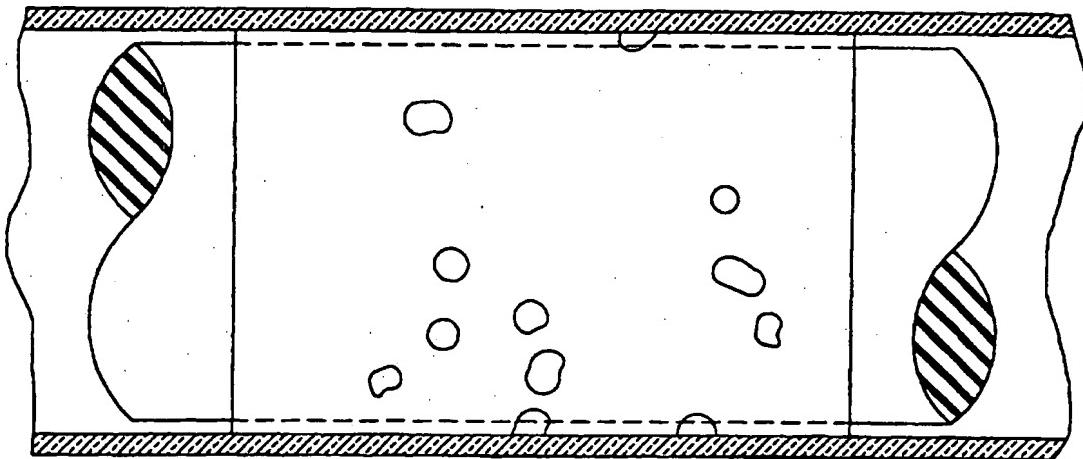


FIG. 3

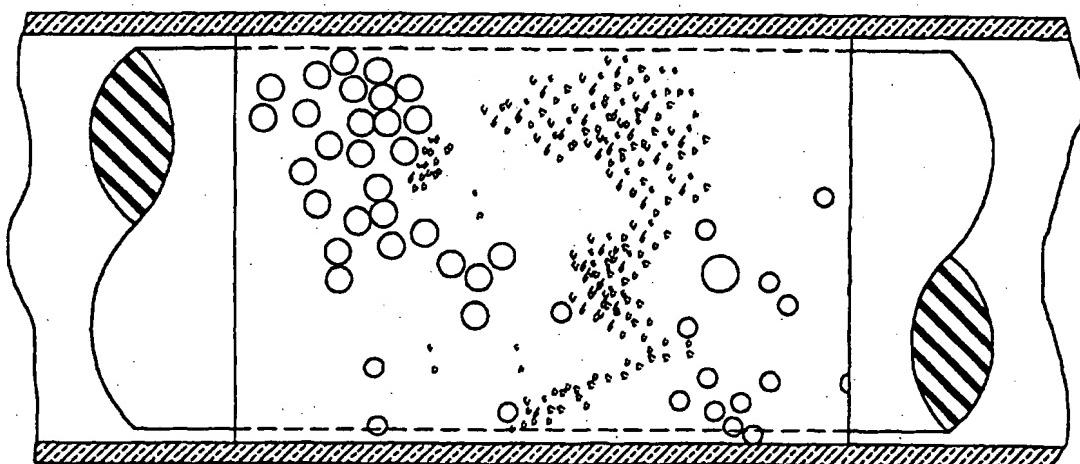


FIG. 4

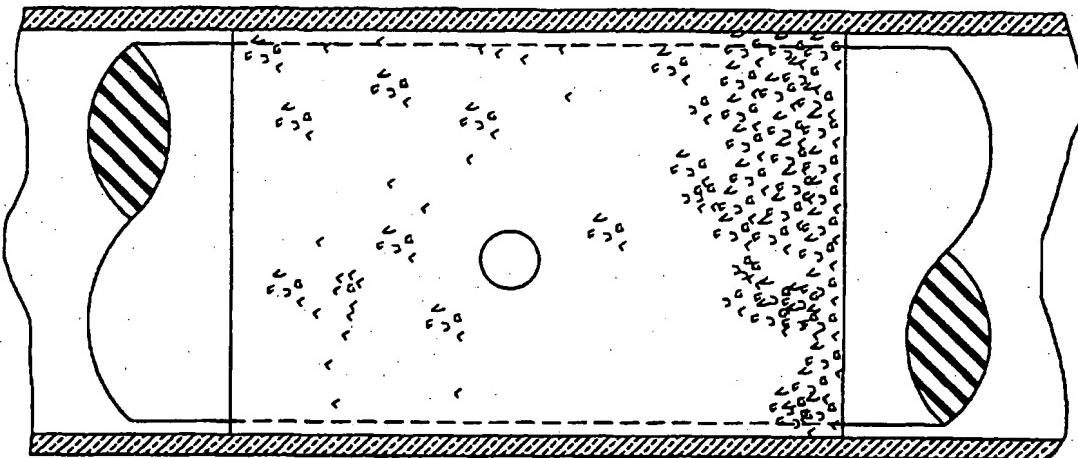


FIG. 5

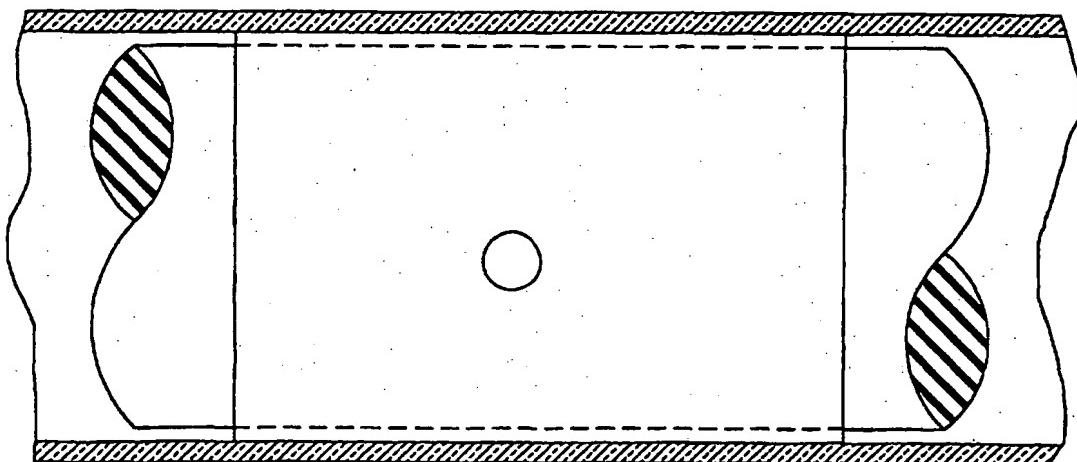


FIG. 6

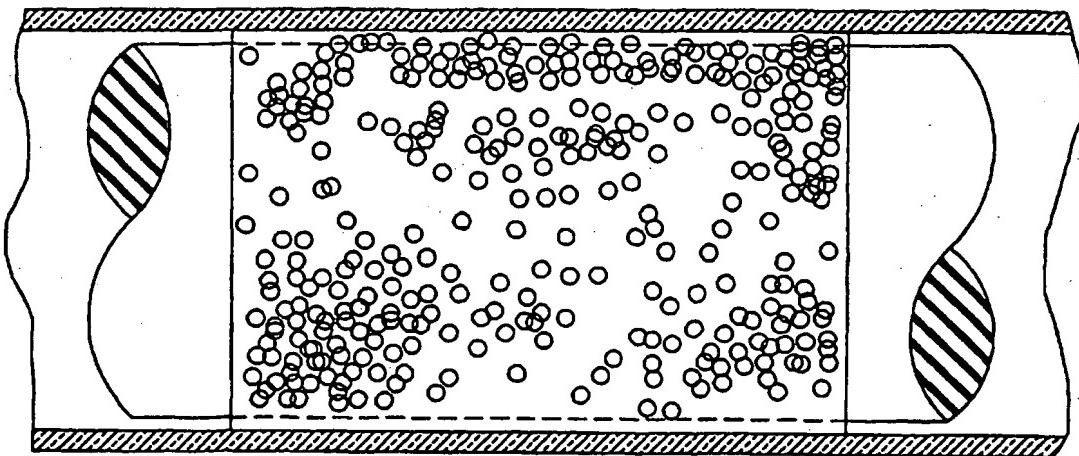


FIG. 7

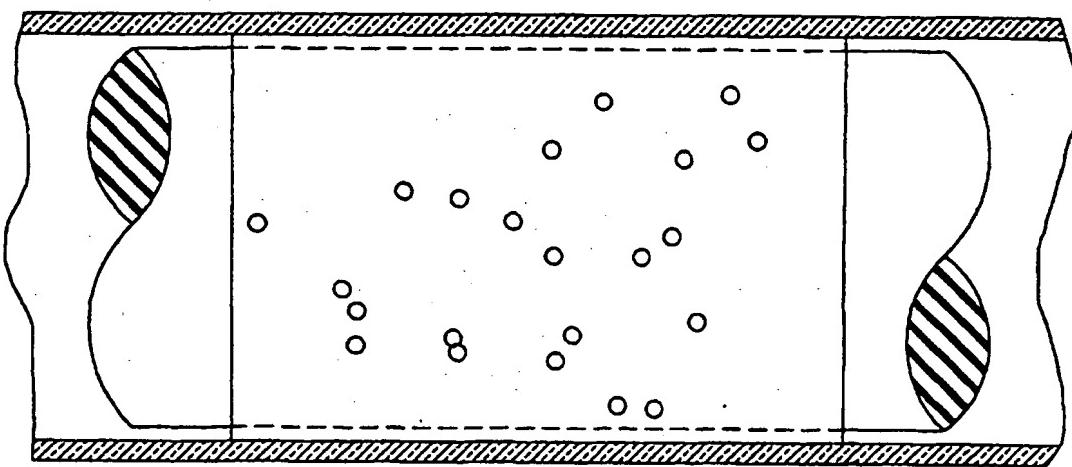


FIG. 8

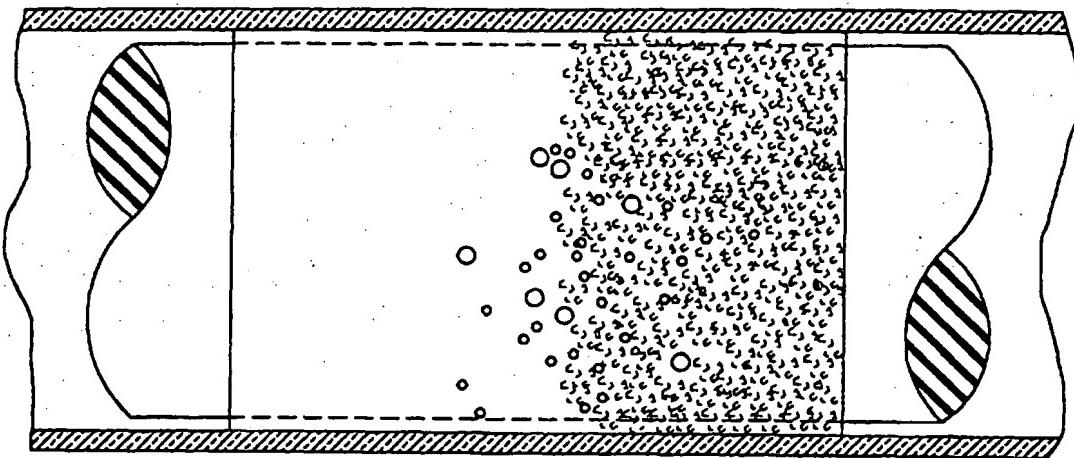


FIG. 9

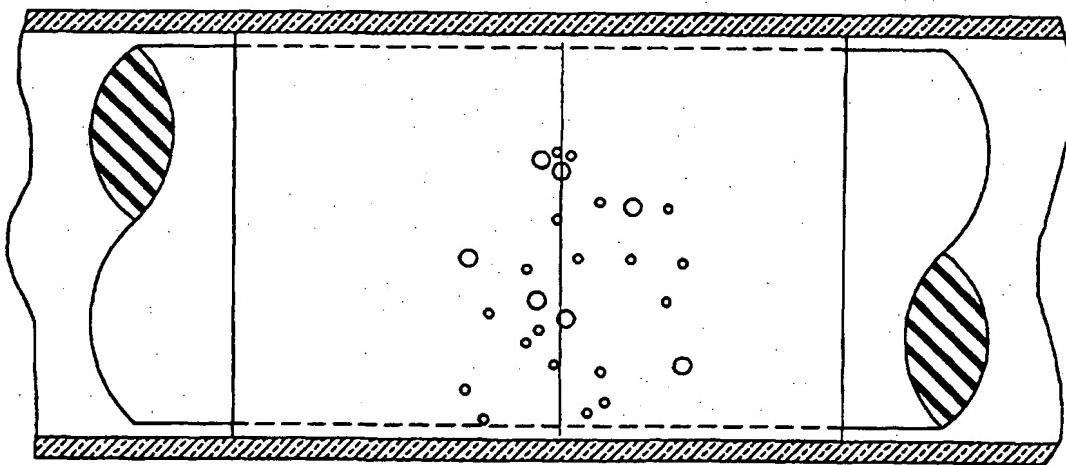


FIG. 10

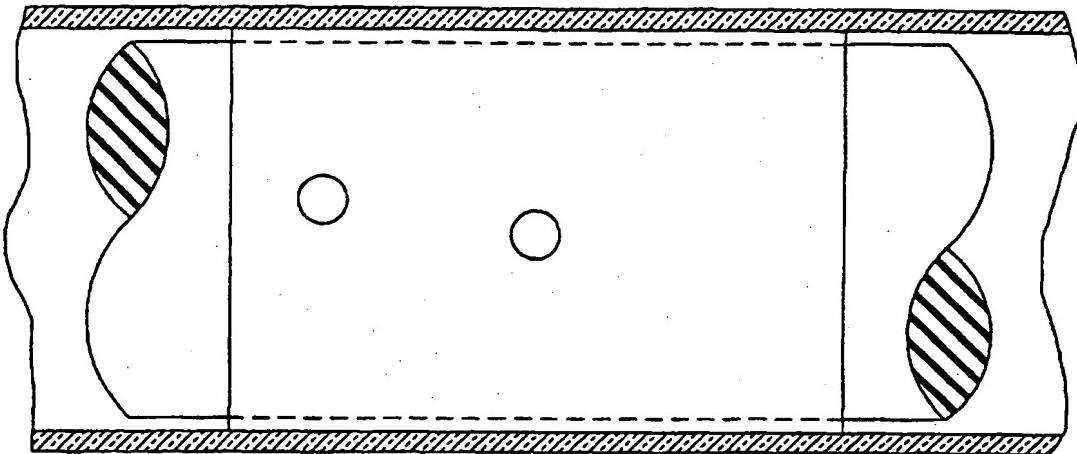


FIG. 11

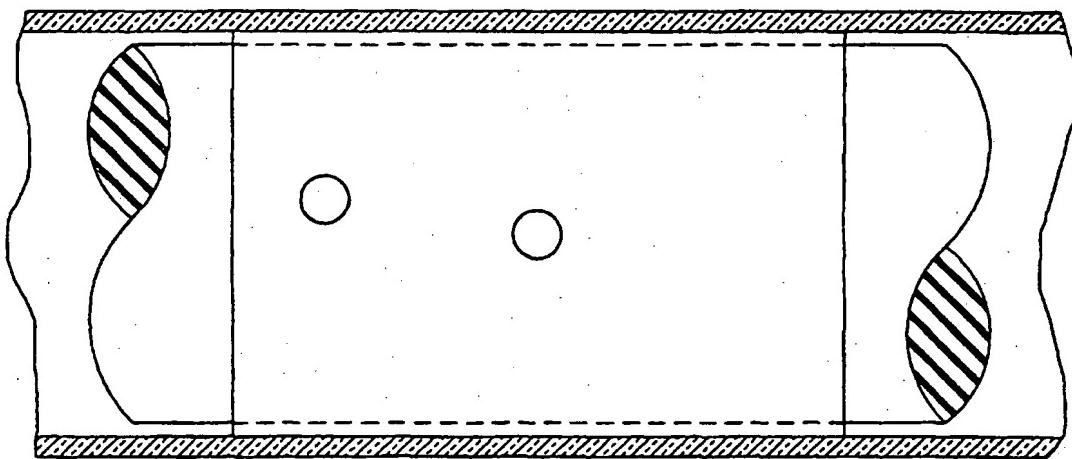
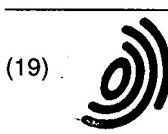


FIG. 12



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(54) MAGNETIC SEPARATION EMPLOYING EXTERNAL AND INTERNAL GRADIENTS

MAGNETISCHE TRENNUNG MIT HILFE VON EXTERNEN UND INTERNEN GRADIENTEN

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Description**Field of the Invention**

5 [0001] The present invention relates to apparatus and methods for separating, immobilizing, and quantifying biological substances from within a fluid medium. More particularly, the invention relates to conducting observations of biological substances by employing a high internal gradient magnetic capture structure formed within a vessel, in conjunction with an externally-applied force for transporting magnetically responsive material toward the capture structure. The present invention is also useful in conducting quantitative analysis and sample preparation in conjunction with automated cell enumeration techniques.

Background of the Invention

15 [0002] A magnetic material or magnetic dipole will move in a magnetic field gradient in the direction of increasing highest magnetic field strength. Magnetic gradients employed in fluid separations are broadly divided into two categories. Internal magnetic gradients are formed by inducing a magnetization in a susceptible material placed in the interior of a separation vessel. External gradients are formed by an externally positioned magnetic circuit.

20 [0003] In the case of a simple rectangular bar magnet, field lines which form magnetic circuits conventionally move from North to South and are easily visualized with iron filings. From this familiar experiment in elementary physics it will be recalled that there is greater intensity of field lines nearest the poles. At the poles, the edges formed with the sides and faces of the bar will display an even greater density or gradient. Thus, a steel ball placed near a bar magnet is first attracted to the nearest pole and next moves to the region of highest field strength, typically the closest edge. For magnetic circuits, any configuration which promotes increased or decreased density of field lines will generate a gradient. Opposing magnet designs, such as N-S-N-S quadrupole arrangements having opposing North poles and 25 opposing South poles, generate radial magnetic gradients.

[0004] Internal high gradient magnetic separators have been employed for nearly 50 years for removing weakly magnetic materials from slurries such as in the kaolin industry or for removing nanosized magnetic materials from solution. In an internal high gradient magnetic separator, a separation vessel is positioned in a uniform magnetic vessel. A ferromagnetic structure is positioned within the vessel in order to distort the magnetic field and to generate an "internal" gradient in the field. Typically, magnetic grade stainless steel wool is packed in a column which is then placed in a uniform magnetic field which induces gradients on the steel wool as in U.S. Patent No. 3,676,337 to Kolm. Gradients as high as 200 kGauss/cm are easily achieved. The magnitude of the field gradient in the vicinity of a wire is inversely related to the wire diameter. The spatial extent of the high gradient region is proportionally related to the diameter of the wire. As will be detailed below, collection of magnetic material takes place along the sides of the wire, perpendicular to the applied magnet field lines, but not on the sides tangent to the applied field. In using such a system, material to be separated is passed through the resulting magnetic "filter". Then, the collected material is washed, and the vessel is moved to a position outside the applied field, so that magnetic can be removed, making the collector ready for reuse.

35 [0005] Table I below indicates the magnitude of a magnetic gradient as a function of distance R, from the center of a ferromagnetic wire for round wires of different diameters. The gradients are determined by Maxwell's equations, which produce equations I and II for the strength of the magnetic field about the wires and the gradient of the field. The equations give the magnitude of these quantities when the wire has an internal magnetization per unit volume of M. If the wires are composed of "soft" ferromagnetic materials, the magnetization depends on the value, B_{ext} , of an externally applied field. For any value of M, even for a hard ferromagnetic material with constant, uniform magnetization, the dependence on the distance and wire diameter are as shown. The gradient values listed in Table 1 assume a typical wire magnetization such that $4\pi M = 10$ kiloGauss (kG), a value close to that of a rare earth magnetic alloy. Table 1 demonstrates that for a narrower wire, the field gradient at the surface of the wire is larger than for thicker wires, although the magnitude of the gradient falls off much more rapidly with distance from the wire.

Table I

| Diameter of wire | | | | | |
|-------------------------------|----------------|----------------|----------------|----------------|----------------|
| | 0.2 μm | 2.0 μm | 20 μm | 200 μm | 2000 μm |
| Distance from wire center (R) | grad B (kG/cm) |
| 0.1 μm | 600,000 | -- | -- | -- | -- |
| 0.2 μm | 75,000 | -- | -- | -- | -- |

Table I (continued)

| | Diameter of wire | | | | |
|----|------------------|-----------|---------|----------|---------|
| | 0.2 μm | 2.0 μm | 20 μm | 200 μm | 2000 μm |
| 5 | 0.5 μm | 4,800 | -- | -- | -- |
| 10 | 1.0 μm | 600 | 60,000 | -- | -- |
| 15 | 2.0 μm | 75 | 7,500 | -- | -- |
| 20 | 5.0 μm | 4.8 | 480 | -- | -- |
| 25 | 10.0 μm | 0.6 | 60 | 6,000 | -- |
| 30 | 20.0 μm | 0.075 | 7.5 | 750 | -- |
| 35 | 50.0 μm | 0.0048 | 0.48 | 48 | -- |
| 40 | 0.10 mm | 0.0006 | 0.06 | 6.0 | 600 |
| 45 | 0.20 mm | 0.000075 | 0.0075 | 0.75 | -- |
| 50 | 0.50 mm | 0.0000048 | 0.00048 | 0.048 | 4.8 |
| 55 | 1.0 mm | | 0.00006 | 0.006 | 0.6 |
| 60 | 2.0 mm | | | 0.00075 | 0.075 |
| 65 | 5.0 mm | | | 0.000048 | 0.0048 |
| 70 | | | | | 0.48 |

$$B_{int} = [B_{ext} (\mu - 1) D^2] / [4 (\mu + 1) R^2] = 2 \pi M D^2 / 4R^2 \quad (I)$$

$$\text{grad } B_{int} = [B_{ext} (\mu - 1) D^2] / [4 (\mu + 1) R^3] = 2 \pi M D^2 / 4R^3 \quad (II)$$

where D = the diameter of a circular wire

R = the distance from the center of the wire

M = the wire magnetization

μ = the magnetic permeability of the wire

B_{ext} = the magnitude of the external field perpendicular to the wire

B_{int} = the magnitude of the resultant internal field contribution

$\text{grad } B_{int}$ = the magnitude of the resultant internal field gradient

[0006] A method and apparatus for separating cells and other fragile particles are described by Graham, et al in U.S. Patent No. 4,664,796. The apparatus contains a rectangular chamber within a cylinder. One pair of opposing sides

of the chamber are made of non-magnetic material, while the other sides are made of magnetic material. The flow chamber is packed with a magnetically responsive interstitial separation matrix of steel wool. The material to be separated is run through the chamber, which is positioned in a uniform magnetic field. During separation, the chamber is aligned in the magnetic field such that the magnetic sides of the chamber are parallel to the applied field lines, thus inducing high gradients about the interstitial matrix in the chamber. When the chamber is in this position, magnetically labeled cells are attracted to the matrix and held thereon, while the non-magnetic components are eluted. The chamber is then rotated, so that the magnetic sides face magnets, which "shunts" or "short-circuits" the magnetic field, reclines the gradients in the flow chamber, and allows the particles of interest to be removed by the shearing force of the fluid flow.

[0007] Other internal magnetic separation devices are known. Commonly owned U.S. Patent No. 5,200,084 teaches the use of thin ferromagnetic wires to collect magnetically labeled cells from solution. U.S. Patent No. 5,411,863 to

Miltenyi teaches the use of coated steel wool, or other magnetically susceptible material to separate cells. U.S. patent application 08/424,271 by Liberti and Wang teaches an internal HGMS device useful for immobilization, observation, and performance of sequential reactions on cells.

[0008] Ahn C.H. et al. (Proceeding of the workshop on micro electro mechanical systems (MEM, OISO, Jan. 25-28, 1994, p. 91-96) describes an apparatus in which microscopic electromagnets are positioned in very small flowthrough systems which are capable of separating particles in a flow of fluid as it passes through the system. The particles are clumped at the pole positions of electromagnets positioned along the walls of channels formed in a substrate.

[0009] WO 94/11078 discloses a magnetic immobilization device in which magnetically-responsive entities are col-

lected along a ferromagnetic capture structure positioned within the interior of a vessel, such as a suspended wire between the end walls of a chamber, or a wire or metallic wedge situated along a side wall of a chamber. In this system, magnetic entities are collected along the edge of the collecting wire or wedge in the interior of the chamber. In order to view the collected entities, it is necessary for the fluid to be non-obscuring because the collected species are positioned at a depth within the chamber and away from the top of the chamber.

[0010] External gradient magnetic separators are also known for collecting magnetically responsive particles. External devices are so-named because in such devices, a high gradient magnetic field is produced by a suitable configuration of magnets positioned external to the separation vessel, rather than by an internal magnetic structure. A standard bar magnet, for example, produces a gradient because the magnetic field lines follow non-linear paths and "fan out" or bulge along respective paths from North to South. Typical gradients of about 0.1 to 1.5 kGauss/cm are produced by high quality laboratory magnets. These relatively low gradients can be increased by configuring a magnetic circuit to compress or expand the field line density. For example, a second bar magnet positioned in opposition to a first magnet causes repulsion between the two magnets. The number of field lines remains the same, but they become compressed as the two magnets are moved closer together. Thus, an increased gradient results. Adding magnets of opposing field to this dipole configuration to form a quadrupole further increases the extent of the high gradient region. Other configurations, such as adjacent magnets of opposing fields, can be employed to create gradients higher than those caused by a bar magnet of equivalent strength. Another method of increasing gradients in external field devices is to vary the shapes of the pole faces or pole pieces. For example, a magnet having a pointed face causes an increased gradient relative to a magnet having a flat pole face.

[0011] U.S. Patent No. 3,326,374 to Jones and U.S. Patent No. 3,608,718 to Aubrey describe typical external gradient separators. Dipole configured separators for preventing scale and lime build up in water systems are described in U. S. Patent No. 3,228,878 to Moody and U.S. Patent No. 4,946,590 to Herzog. Adjacent magnets of opposing polarity have been used in drum or rotor separators for the separation of ferrous and non-ferrous scrap, as described in U.S. Patent No. 4,869,811 to Wolanski et al. and U.S. Patent No. 4,069,145 to Sommer et al.

[0012] External gradient devices have also been used in the fields of cell separation and immunoassay. U.S. Patent Nos. 3,970,518 and 4,018,886 to Giaever describe the use of small magnetic particles to separate cells using an actuating coil. Dynal Corp. (Oslo, Norway) produces separators employing simple external magnetic fields to separate carrier beads for various types of cell separations. Commonly owned U.S. Patent Nos. 5,466,574 and 5,541,072 disclose the use of external fields to separate cells for solution to form a monolayer of cells or other biological components on the wall of a separation vessel. Resuspension and recovery of the collected material usually requires removal of the collection vessel from the gradient field and some level of physical agitation. Turning now to the magnetic particles used in such collection devices, superparamagnetic materials have in the last 20 years become the backbone of magnetic separations technology in a variety of healthcare and bioprocessing applications. Such materials, ranging in size from 25 nm to 100 μm , have the property that they are only magnetic when placed in a magnetic field. Once the field is removed, they cease to be magnetic and can be redispersed into suspension. The basis for superparamagnetic behavior is that such materials contain magnetic cores smaller than 20-25 nm in diameter, which is estimated to be less than the size of a magnetic domain. A magnetic domain is the smallest volume for a permanent magnetic dipole to exist. Magnetically responsive particles can be formed about one or more such cores. The magnetic material of choice is magnetite, although other transition element oxides and mixtures thereof can be used.

[0013] Magnetic particles of the type described above have been used for various applications, particularly in health care, e.g. immunoassay, cell separation and molecular biology. Particles ranging from 2 μm to 5 μm are available from Dynal. These particles are composed of spherical polymeric materials into which magnetic crystallites have been deposited. These particles because of their magnetite content and size, are readily separated in relatively low external gradients (0.5 to 2 kGauss/cm). Another similar class of materials are particles manufactured by Rhone Poulenc which typically are produced in the 0.75 μm range. Because of their size, they separate more slowly than the Dynal beads in equivalent gradients. Another class of material is available from Advanced Magnetics. These particles are basically clusters of magnetite crystals, about 1 μm in size, which are coated with amino polymer silane to which bioreceptors can be coupled. These highly magnetic materials are easily separated in gradients as low as 0.5 kGauss/cm. Due to their size, both the Advanced Magnetics and Rhone Poulenc materials remain suspended in solution for hours at a time.

[0014] There is a class of magnetic material which has been applied to bioseparations which have characteristics which place them in a distinct category from those described above. These are nanosized colloids (see U.S. Patent Nos. 4,452,773 to Molday; 4,795,698 to Owen, et al; 4,965,007 to Yudelson; 5,512,332 to Liberti & Piccoli; 5,597,531 to Liberti, et al and U.S. Patent Application 08/482,448 to Liberti, et al). They are typically composed of single to multi crystal agglomerates of magnetite coated with polymeric material which make them aqueous compatible. Individual crystals range in size from 8 to 15 nm. The coatings of these materials have sufficient interaction with solvent water to keep them permanently in a colloidal suspension. Typically, well coated materials below 150 nm will show no evidence of settling for as long as 6 months. These materials have substantially all the properties of ferrofluids.

[0015] Because of the small particle size and strong interaction with solvent water, substantial magnetic gradients

are required to separate ferrofluids. It had been customary in the literature to use steel wool column arrangements described above which generate 100-200 kGauss/cm gradients. However, it was subsequently observed that such materials form "chains" (like beads on a string) in magnetic fields, thus allowing separation in gradient fields as low as 5 or 10 kGauss/cm. This observation led to development of separation devices using large gauge wires which generate relatively low gradients. Large gauge wires can be used to cause ferrofluids to produce uniform layers upon collection. By controlling amounts of ferrofluid in a system, a monolayer can be formed. Magnetically labeled cells can thus be made to form monolayers as described in commonly owned U.S. Patent Nos. 5,186,827 and 5,466,574.

[0016] Analysis of the cellular composition of bodily fluids is used in the diagnosis of a variety of diseases. Microscopic examination of cells smeared or deposited on slides and stained by Romanowsky or cytochemical means has been the traditional method for cell analysis. Introduction of impedance based cell counters in the late 1950s has led to a major advance in the accuracy of cell enumeration and cell differentiation. Since then, various other technologies have been introduced for cell enumeration and differentiation such as Fluorescence Activated Flowcytometry, Quantitative Buffy Coat Analysis, Volumetric Capillary Cytometry and Laser Scanning Cytometry. Fluorescence based flowcytometry has improved the ability to discern different cell types in heterogeneous cell mixtures. Simultaneous assessment of multiple parameters of individual cells which pass a measurement orifice at a speed of up to 1,000 to 10,000 cells/sec is a powerful technology. However, there are limitations of the technology, such as an inability to analyze high cell concentration requiring dilution of blood, impracticability of detecting of infrequent or rare cells, and an inability to reexamine cells of interest. To overcome these limitations, clinical samples are typically subjected to various enrichment techniques such as erythrocyte lysis, density separation, immunospecific selection or depletion of cell populations prior to analysis by flowcytometry.

[0017] Many bioanalytical techniques involve identification and separation of target entities such as cells or microbes within a fluid medium such as bodily fluids, culture fluids or samples from the environment. It is also often desirable to maintain the target entity intact and/or viable upon separation in order to analyze, identify, or characterize the target entities. For example, to measure the absolute and relative number of cells in a specific subset of leukocytes in blood, a blood sample is drawn and incubated with a probe, for example a fluorescently labeled antibody specific for this subset. The sample is then diluted with a lysing buffer, optionally including a fixative solution, and the dilute sample is analyzed by flow cytometry. This procedure for analysis can be applied to many different antigens. However, the drawbacks to this procedure become apparent when large samples are required for relatively rare event analyses. In those situations, the time needed for the flow cytometer to analyze these samples becomes extremely long, making the analysis no longer feasible due to economic concerns.

[0018] One system which attempted to overcome some of the problems with flow cytometers was the so-called "Cytodisk," described in 1985 by DeGrooth, Geerken & Greve (*Cytometry*, 6: 226-233 (1985)). The authors describe a method of aligning cells in the grooves of a gramophone disk. The disk with dried cells was placed on a record player, and the arm of the record player was outfitted with an optical fiber immediately behind the needle. The needle kept the optical fiber aligned with the grooves in the record. The unicellular algae cells (3 microns in diameter) used in the reported experiment remained in the bottom of the groove, awaiting analysis by the optical system. The advantages of the Cytodisk included that cells could be subjected to multiparameter measurement with no optical cross-talk, individual cells could be indexed to said measurements, and cells could be measured repeatedly at different levels of analytical resolution. However, the system required that the cells be dried upon the gramophone record, a nonhomogeneous process damaging to many cells. Even if cells were effectively dried upon the record for analysis, they would be dead cells. The current invention seeks to combine some of the benefits provided by the Cytodisk, including multiparameter measurement, indexing, and repeated measurement with new features which allow analysis of intact cells, which can be released for culturing or other re-use, including infusion back into a living organism.

45 SUMMARY OF THE INVENTION

[0019] This invention relates to the immobilization of microscopic entities, including biological entities, such as cells which enables separation of such entities from a fluid medium, including whole blood, into a defined region in a collection chamber, such that analysis by automated means is possible. This invention also provides for the quantitative collection of magnetically labeled target entities, such that microliter quantities of sample can be used to detect target entities, including those entities which occur at low frequencies.

[0020] In a preferred embodiment of the invention, a collection vessel is provided in which ferromagnetic lines are supported by adhesion along a transparent wall. The lines have effective diameters of 0.1 μm to 30 μm , resulting in immobilization and alignment of magnetically labeled biological materials in an ordered array. In a particularly preferred embodiment of the invention, human blood cells are aligned for automated analysis.

[0021] The methods of the invention employ dual forces for collecting particles. In one embodiment, target material is brought into range of the internal high gradient region by gravity. In another embodiment, a single applied magnetic field serves dual purposes. The applied field comprises a first, external magnetic gradient which moves magnetically

responsive particles to a region of a collection vessel. At the same time, the applied field induces magnetization in a ferromagnetic collection structure, thereby adding a second, internal gradient which further acts upon the magnetically responsive particles to move them into defined region of the collection vessel for analysis. The vessel may be oriented such that the external gradient acts in opposition to, or in conjunction with, the influence of gravity upon the target material.

[0022] The methods of the invention have utility in the separation of biological entities which include a wide variety of substances of biological origins including cells, both eukaryotic (e.g. leukocytes, erythrocytes, platelets, epithelial cells, mesenchymal cells, or fungi) and prokaryotic (e.g. bacteria, protozoa or mycoplasma), viruses, cell components, such as organelles, vesicles, endosomes, lysosomal packages or nuclei, as well as molecules (e.g. proteins) and macromolecules (e.g. nucleic acids - RNA and DNA). The biological entities of interest may be present in at least samples or specimens of varying origins, including, biological fluids such as whole blood, serum, plasma, bone marrow, sputum, urine, cerebrospinal fluid, amniotic fluid or lavage fluids, as well as tissue homogenates, disaggregated tissue, or cell culture medium. They may also be present in material not having a clinical source, such as sludge, slurries, water (e.g. ground water or streams), food products or other sources. The method of the invention also has utility in the separation of various bacteria and parasites from fecal matter, urine, or other sources.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023]

FIG. 1 is an exploded perspective view of a separation vessel according to one embodiment of the invention; FIG. 2 is an enlarged perspective view of a ferromagnetic grid disposed in the separation vessel of FIG. 1; FIG. 3 is a side elevational view of an arrangement for viewing collected cells in the separation vessel of FIG. 1; FIGS. 4A and 4B are photographs of cells collected in the arrangement of FIG. 3 under alternate methods of illumination; FIG. 5A is a perspective view of an alternative embodiment of a separation vessel containing a ferromagnetic capture structure according to the present invention; FIG. 5B is a sectional view of the separation vessel taken along the line 5B-5B of FIG. 5A; FIG. 6 is a schematic diagram of the separation vessel of FIG. 5A positioned in a relatively homogenous horizontal external magnetic field; FIG. 7 is a computer generated diagram of the effect of the applied magnetic field of FIG. 6 upon movement of individual magnetically responsive particles disposed throughout the separation vessel; FIG. 8 is a schematic diagram of the separation vessel positioned in an applied field having an external gradient; FIG. 9 is a computer generated diagram of the path component of numerous magnetically responsive particles in the separator of FIG. 8 due solely to the external gradient; FIG. 10 is a computer generated diagram of the paths followed by magnetically responsive particles in the separation vessel of FIG. 8, taking into account the external gradient, the internal gradient, and gravity; FIG. 11 is a schematic diagram an arrangement for automated analysis of collected target entities within a separation vessel of the type shown in FIG. 5A; and FIG. 12 is a schematic diagram of a separation vessel for use in performing sequential reactions and analysis target material captured therein.

DETAILED DESCRIPTION OF THE INVENTION

45 I. General Definitions

[0024] Unless otherwise indicated, terms of general usage throughout the present specification are defined as follows.

[0025] The term "probe" as used herein refers to an antibody or other specific binding substance which contains or is adapted to include a detectable label. Detectable labels include fluorescent, chemiluminescent and radioactive compounds, as well as compounds which have distinct or recognizable lightscattering or other optical properties. Detectable labels also include those compounds which are only detectable upon binding to the characteristic determinant.

[0026] The term "ferromagnetic capture structure" as used herein refers to a structure of ferromagnetic material which becomes magnetized in the presence of a magnetic field to attract magnetically responsive particles. The capture structure may be provided in the form of wires, thin strips, lithographically formed strips, or electroplated ferromagnetic material supported on or by a wall of a separation vessel. The ferromagnetic material may include iron, nickel, cobalt, alloys of the same, alloys of magnetic rare earth elements, or other paramagnetic materials. The term "internal gradient" as used herein refers to a magnetic gradient induced by the capture structure when it is placed in a magnetic field.

The term "external gradient" refers to a magnetic gradient applied solely by a configuration of magnets or pole pieces, external to the separation vessel. Electromagnets can also be used to form magnetic fields useful in the invention.

[0027] The term "determinant" is used here in a broad sense to denote that portion of the biological entity involved in and responsible for selective binding to a specific binding substance, the presence of which is required for selective binding to occur. The expression "characteristic determinant" is used herein in reference to cells, for example, to signify an epitope (or group of epitopes) that serve to identify a particular cell type and distinguish it from other cell types. Cell-associated determinants include, for example, components of the cell membrane, such as membrane-bound proteins or glycoproteins, including cell surface antigens of either host or viral origin, histocompatibility antigens or membrane receptors.

[0028] The expression "specific binding substance" as used herein refers to any substance that selectively recognizes and interacts with the characteristic determinant on a biological entity of interest, to substantial exclusion of determinants present on biological entities that are not of interest. Among the specific binding substances which may be used in affinity binding separations are antibodies, anti-haptens, lectins, peptides, peptide-nucleic acid conjugates, nucleic acids, Protein A, Protein G, concanavalin A, soybean agglutinin, hormones and growth factors. The term "antibody" as used herein includes immunoglobulins, monoclonal or polyclonal antibodies, immunoreactive immunoglobulin fragments, single chain antibodies, and peptides, oligonucleotides or any combination thereof which specifically recognize determinants with specificity similar to traditionally generated antibodies.

[0029] The term "magnetically responsive particles" as used herein refers to magnetic particles of metallic or organometallic composition, optionally coated with polymer, preferably coated with a polymer of biological origin such as BSA. The particles may be linked with an antibody or other specific binding substance to allow them to bind to biological entities of interest. Appropriate magnetic material is manufactured by Dynal, Rhone Poulenc, Miltenyi Biotec, Cardinal Associates, Bangs Labs, Ferrofluidics, and Immunicon Corp. Also included in the term "magnetically responsive particles" is a biological entity-magnetic particle complex, optionally bound to a fluorescent label or other detectable label.

[0030] The preferred magnetic particles for use in carrying out this invention are particles that behave as true colloids. Such particles are characterized by their sub-micron particle size, which is generally less than about 200 nanometers (nm), and their stability to gravitational separation from solution for extended periods of time. Such small particles facilitate observation of the target entities via optical microscopy since the particles are significantly smaller than the wavelength range of light. Suitable materials are composed of a crystalline core of superparamagnetic material surrounded by molecules which may be physically absorbed or covalently attached to the magnetic core and which confer stabilizing colloidal properties. The size of the colloidal particles is sufficiently small that they do not contain a complete magnetic domain, and their Brownian energy exceeds their magnetic moment. As a consequence, North Pole, South Pole alignment and subsequent mutual attraction/repulsion of these colloidal magnetic particles does not appear to occur even in moderately strong magnetic fields, contributing to their solution stability. Accordingly, colloidal magnetic particles are not readily separable from solution as such even with powerful electromagnets, but instead require a magnetic gradient to be generated within the test medium in which the particles are suspended in order to achieve separation of the discrete particles. Magnetic particles having the above-described properties can be prepared as described in U.S. Patent Nos. 4,795,698, 5,512,332 and 5,597,531.

II. Gravitationally-Assisted Internal Gradient Immobilization

[0031] Referring now to FIG. 1, there is shown an exploded view of a separation vessel 10 according to a first embodiment of the invention. The vessel 10 comprises a pair of opposed parallel wall members 12 and 14 separated by perpendicular walls 16 defining an interior chamber. A ferromagnetic collection structure comprising a plurality of longitudinally extensive members is supported on and by an interior surface of the chamber. For example, a nickel mesh 18 is positioned upon the interior surface of the wall 14 and held thereon by an adhesive.

[0032] A portion of the mesh 18 is shown in FIG. 2. The mesh is formed by electroplating techniques, so that there are no interwoven or overlapping intersections that would undesirably entrap non-target substances by capillary attraction. Suitable meshes include nickel grids used in electron microscopy and are sold by Polysciences, Inc. of Warminster, Pennsylvania (for example, catalog # 8424N). The mesh comprises a plurality of longitudinal members 18a joined to cross members 18b forming a grid for mechanical support. The separation between the longitudinal members 18a should be at least twice the diameter of the particles desired to be collected. When the vessel 10 is positioned in a magnetic field transverse to the longitudinal members 18a, magnetically-labeled target material will be captured along both sides of each of the longitudinal members 18a. In order to form a monolayered linear array of the target particles along the interior surface of the wall 14 supporting the mesh 18, the height of the longitudinal members 18a should be no greater than the average diameter of the target entities. The number of target entities that may be captured in the vessel 10 is equal to twice the total length of the longitudinal members 18a divided by the average diameter of the target particles. The ferromagnetic collection structure, and hence the chamber, can thus be sized to permit collection of substantially all of the target entities expected to be present in a sample of test fluid.

EXAMPLE 1**Leukocyte Differentiation in Whole Blood**

- 5 [0033] A vessel 10 was constructed having longitudinal members 18a extending 150 mm along a region of the wall 14 measuring 5 mm by 3 mm. The longitudinal members 18a were 5 μm in height, 20 μm wide, and separated by 63 μm spaces. The supporting members 18b were 48 μm wide. The height of the chamber was 0.13 mm, for a chamber volume of 2 μl . Hence, for collecting leukocytes, which have an average diameter of 10 μm , the particle collection capacity was 30,000. Such a collection capacity is sufficient for collecting substantially all leukocytes in the chamber volume.
- 10 [0034] A test fluid was prepared by adding 0.4 μg of a 130 nm CD45-labeled ferrofluid, 3 ng of acridine orange, and 10 ng of ethidium bromide to 1 μl of blood. The test fluid was allowed to incubate for 10 minutes, and deposited in the vessel 10. The vessel was then placed between a pair of magnets 20a and 20b, as shown in FIG. 3, with the mesh 18 positioned at the bottom of the chamber to allow the labeled cells to settle toward the mesh under the influence of gravity, and then to be aligned along the longitudinal members of the mesh in the internally-generated high gradient regions along the longitudinal members. To improve visibility of the captured material, the arrangement shown in FIG. 3 can then be inverted to allow the non-target material to settle away from the mesh 18.
- 15 [0035] Acridine orange is absorbed by the nucleated cells, which will emit green light when excited by blue light (460 - 500 nm). Under the same illumination, intracytoplasmatic granules of granulocytes will emit red light. Ethidium bromide is absorbed only by cells having non-intact membranes (i.e. dead cells), and will emit deep red light under blue illumination. The optical response of the material in the chamber to blue illumination was viewed through an inverted microscope 22. The resulting photographic image of FIG. 4A was obtained under a combination of blue illumination (permitting visibility of the fluorescence emitted from the captured cells) and ambient white illumination (permitting visibility of the ferromagnetic mesh). The captured fluorescent cells can easily be distinguished from other blood components.
- 20 [0036] Discrimination between cell types can be achieved by detection of selected emission spectra and lightscatter properties of the collected cell. It will be apparent to those skilled in the art that probes with various specificities and different fluorescence excitation and emission can be used to differentiate between the captured, aligned cells. Also, one or more excitation wavelengths can be used to discriminate between the targets, or between the targets and the collection structure. For example, FIG. 4B shows the same collected cells under monochromatic blue fluorescent light. The collection structure is no longer easily visible, and the collected leukocytes are readily discernible. A standard microscope could then be used to observe the cells.
- 25 [0037] This example illustrates the differentiation of two types of cells. In this case, leukocytes were separated from other cell types and among the aligned leukocytes, live cells were discriminated from dead cells by the use of dyes. It will be apparent to one skilled in the art that any two (or more) cell types can be differentiated using different probes.
- 30 [0038] For example: fetal nucleated red blood cells from maternal blood, circulating tumor cells (Epcam $^+$ CD45 $^+$) from normal nucleated blood cells, platelets (CD41 $^+$, PAC-1 $^+$) from activated platelets (CD41 $^+$, PAC-1 $^+$), and various leukocyte subsets. Important leukocytes subsets found in human blood include CD4 $^+$ or CD8 $^+$ cells (T-lymphocyte cells); CD56 $^+$ cells (NK cells); CD19 $^+$ cells (B-lymphocytes); CD14 $^+$ cells (monocytes); CD83 $^+$ cells (dendritic cells); CD33 $^+$, CD66a $^+$, or CD64 $^+$ cells (granulocytes); CD66a $^+$ CD66b $^+$ cells (activated granulocytes); CD34 $^+$ cells (progenitor cells); and CD90w $^+$ cells (hematopoietic stem cells).

EXAMPLE 2**Immunophenotypic Differentiation in Whole Blood**

- 45 [0038] A blood sample is incubated with a fluorescent nucleic acid dye and ferrofluid labeled with an antibody directed against a cell surface epitope such as, for instance, CD4 expressed on T-helper lymphocytes and monocytes or CD34 present on progenitor cells. The incubation can take place in or outside the separation vessel. The vessel is then introduced into a magnetic field and the cells exhibiting the cell surface antigen recognized by the bioactive ferrofluid align on both sides of the ferromagnetic lines. Although all nucleated cells are fluorescently labeled, only those which are adjacent to the ferromagnetic lines are target cells and will be identified as such by an optical detection system arranged to scan for cells along the lines, as described further herein.
- 50 [0039] When the target cell frequency is low, as is the case for progenitor cells in peripheral blood identified by CD34 in normal donors (1-10 CD34 $^+$ cells/ μl), the likelihood increases that non-target cells present by coincidence along the ferromagnetic lines will influence the accuracy of the enumeration. The likelihood that non-target cells are present by coincidence along a ferromagnetic line, and thus mistakenly enumerated as a target cell, can be reduced by decreasing the total length of the ferromagnetic lines. This can be achieved by decreasing the number of ferromagnetic lines in the chamber.

[0040] An alternative approach is not to use a fluorescent nucleic dye but to use a fluorescent labeled monoclonal antibody or other probes directed against the same cell type as the bioactive ferrofluid. Preferably this probe is directed against a different epitope as the bioactive ferrofluid. In this approach only the target cells are fluorescently labeled and identified as such. A drawback of the latter procedure is, however, that it requires a higher sensitivity of the detection system. Other labelling strategies include those generally used in flowcytometry and referred to as multi-color and/or multidimensional analysis. In this case, a bioactive ferrofluid is used to align the particles of interest along the ferromagnetic lines and a variety of monoclonal antibodies or other antigen specific probes labeled with different fluorochromes are used to identify different characteristics or populations within the immunomagnetically immobilized particles.

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III. External Field-Aided Internal Gradient Immobilization

[0041] Separation methods according to a second embodiment of the invention employ both internally-generated and externally-applied magnetic gradients for collecting and immobilizing magnetically-responsive target substances. 15 A non-uniform magnetic field is applied to a separation vessel. The external magnetic gradient moves magnetically responsive particles towards a ferromagnetic capture means. The applied magnetic field also induces magnetization of a ferromagnetic capture structure supported in the vessel. As the magnetically responsive particles move towards the ferromagnetic capture structure, they experience the additional influence of the internally-generated gradient, and are drawn toward the capture structure. If the capture structure is of an appropriate configuration, magnetically responsive particles are immobilized to align along the capture structure, and can be analyzed through a transparent wall of a chamber defined by the separation vessel. If the target material is appropriately labeled, fluorescence or light scattering can be measured through the wall to quantify the amount of target material in the test sample.

[0042] In a preferred embodiment of the invention, the applied magnetic field impels movement of the magnetically responsive particles against the force of gravity, providing an additional means of separation of labeled from unlabeled 20 particles, thus reducing non-specific collection of particles.

[0043] Target substances labeled with the magnetically responsive colloidal particles described above can be collected in a collection vessel 10, shown in FIGS. 5A and 5B. The vessel 10 comprises a tub-shaped carrier member 12 having a recess formed therein, and a top wall member 14. The wall member 14 is configured to fit into the carrier member 12 to define a chamber 11 bounded by the interior surface of the wall member 14 and the interior surfaces of 30 the carrier member 12. The wall member 14 is formed of a non-magnetic transparent material, such as glass, quartz or clear plastic. The carrier member 12 is also formed of a non-magnetic material, and is preferably also transparent.

[0044] The wall member 14 covers a portion of the recess formed in carrier member 12 to provide orifices 16a and 16b at opposed longitudinal ends of the chamber 11. The exposed recessed portions 18a and 18b of the carrier member 12 provide receptacles into which a drop of test fluid may be placed for analysis. Such a fluid may then flow into the 35 chamber 11. Entry of fluid into the chamber 11 can be enhanced by capillary action, if the chamber is sufficiently narrow. Fiducial reference marks (not shown) may be formed or imprinted upon the wall member 14 to provide means for measuring the volume of fluid contained within the chamber 11.

[0045] A ferromagnetic collection structure is supported by adhesion or formed upon the interior surface of the wall member 14. In the embodiment shown in FIGS. 5A and 5B, the ferromagnetic collection structure comprises a plurality of lithographically-defined ferromagnetic lines 20 formed upon the interior surface of the wall member 14. The walls of the chamber 11 are optionally coated with a material such as BSA, silicone, or a negatively charged surface coating to provide chemically or biologically inert exposed interior surfaces. It is important to eliminate a buildup of electrostatic charge upon the wall surfaces to limit non-specific binding of target particles or free magnetic material to the walls of the chamber.

[0046] When the vessel 10 is placed into a magnetic field, the ferromagnetic lines 20 will become magnetized. The 45 magnetic gradients produced by such lines 20 are comparable to the gradients calculated for a circular wire having the same cross-sectional area. The width of the ferromagnetic lines will not affect the monolayering of the particles along the magnetic lines, but the width does affect the strength of the magnetic gradient. The gaps between the lines 20 are preferably at least twice the diameter of the target particles. Optionally, a single line may be used for collection.

[0047] The thickness of the magnetic lines is chosen to be on the order of magnitude of the magnetically responsive 50 target entities to be collected, so that the target entities will align along opposite sides 7 of the lines in a monolayer. Therefore, the lines 20 may be on the order of thickness of the particles to be collected. Preferably, the lines 20 are thinner than the diameter of the entities to be collected. If the entities to be collected are human lymphocytes on the order of 10 μm in diameter and the ferromagnetic lines 20 are about 5 μm thick, the cells will align in a single layer about 10 μm thick. It is particularly preferred for the magnetic lines to be significantly thinner than the diameter of particles to be collected. For example, magnetic lines on the order of 0.25 μm may be used to collect entities of 10 μm in diameter.

[0048] Such thin ferromagnetic lines can be manufactured by methods currently used in the manufacture of computer

5 chips. In such a method, the surface of the wall member is first coated with a ferromagnetic material by a vapor deposition technique, such as vacuum evaporation or sputtering. Such a technique provides a layer of metal adhered to the eventual interior surface of the vessel. The combination of ferromagnetic material and the material used to form the wall member should be selected to provide sufficient adhesion of the ferromagnetic material to the wall member.

10 A layer of photosensitive polymer, or photoresist, is then applied to the coated surface of the wall member and exposed to a pattern of ultraviolet light corresponding to the desired pattern of the ferromagnetic capture structure (or a negative image thereof, depending upon the photoresist employed). The photoresist is then developed to render undesired portions of the metal coating susceptible to removal by etching, such as wet chemical etching or reactive ion etching. Alternatively, the lines may be formed by a lift-off procedure wherein a photoresist pattern is first applied to the wall member and is removed subsequent to deposition of a ferromagnetic coating.

15 [0049] Such lithographic methods may be employed to produce a selected pattern of ferromagnetic metallization on a single wall member or upon a large-area substrate that is later to be divided into a plurality of wall members. These lithographic techniques can be substantially cheaper than the use of electroplating or electroforming, which would be used for thicker lines. Thin lines, by their nature, also tend to be smoother than their thicker counterparts. Such consistency is a by-product of the manufacturing technique. Smoother lines are important, because the induced magnetic fields are likewise more consistent. Since such small lines are being used, the strength of the magnetic field will vary greatly along a relatively "bumpy" line, which will lead to clumping of the collected magnetic material. Thus, a smoother ferromagnetic capture structure and more consistent magnetic fields will result in more evenly spaced magnetic material, facilitating automated examination of the collected material.

20 [0050] The separation of a magnetically responsive target substance, using the vessel 10, shall now be described in connection with various magnetic arrangements wherein an exemplary target substance shall be human lymphocytes labeled with magnetic particles manufactured as described in U.S. Patent Nos. 4,795,698, 5,512,332 and 5,597,531 and in U.S. Patent Application No. 08/482,448.

25 [0051] FIG. 6 shows the vessel 10 positioned in a substantially uniform magnetic field, shown by field lines 30, created in a gap between two magnets 21 of opposing polarity. For proper magnetization, the longitudinal axis of the ferromagnetic capture structure is oriented perpendicular to the field lines 30.

30 [0052] FIG. 7 is a computer-generated diagram illustrating the paths 40 followed by numerous magnetically responsive particles 41 after the vessel is positioned in a homogeneous magnetic field. Magnetized ferromagnetic lines 20 with a thickness of 5 μm are shown end-on. A majority of particles in the chamber are unaffected by the internal magnetic gradients and eventually fall to the bottom of the chamber under the influence of gravity.

35 [0053] In the computer simulation employed to produce Fig. 7, all of the particles in the chamber were assumed to be magnetically responsive. In an actual separation, the majority of cells will not be magnetically responsive, and will thus settle to the bottom of the chamber under the influence of gravity. The relatively few target cells will collect in respective linear monolayers along the ferromagnetic lines.

40 [0054] In order to obtain quantitative information about the target particles, a reproducible and high percentage of the particles would desirably be collected by the device. In order to obtain information about relatively rare events, such as circulating tumor cells, fetal cells in maternal blood, or hematopoietic stem cells, virtually all target particles must be collected. In order to use relatively thin ferromagnetic structures to obtain alignment of the particles, a method of "sweeping up" the cells in the chamber is necessary to move the particles into the spatially limited internal high gradient regions. One way of "sweeping up" particles would be to use a narrow chamber. As indicated in Fig. 7, a chamber thickness of just under 100 μm is sufficient for 5 μm ferromagnetic lines, but this would require a small chamber volume, which would limit the opportunity to observe rare species. Using a long chamber to increase volume would require a longer ferromagnetic capture structure, and would increase the time needed to search along the capture structure for the collected target material. One could alternatively turn the chamber upside-down, such that gravity would assist to settle all particles upon the wires as described above in connection with the first embodiment. However, in fluids having a heterogeneous population dominated by non-target species, magnetically labeled material may not be able to move through a thick layer of settled non-target material to reach the ferromagnetic capture structure, resulting in loss of selectivity and crowding of the detection area. Another approach would be to increase the field strength of the magnets, but as shown in formula II, to double the range of the gradient, one would have to increase the external field strength eight-fold.

45 [0055] One method of the instant invention uses a non-uniform applied magnetic field to magnetize the ferromagnetic capture structure and also to provide an external gradient perpendicular to the capture structure "sweep up" the magnetically responsive particles not initially located within the influence of the internal magnetic gradients. The applied magnetic field preferably supplies an external gradient of sufficient magnitude to transport the cells towards the ferromagnetic capture structure where they are then immobilized against the wall adjacent the capture structure by the internal magnetic gradient. Attributes of such a field include that it is substantially homogeneous within a plane parallel to the ferromagnetic capture structure, and that the field is oriented perpendicular to the horizontal longitudinal axis of the structure. Additionally, the field includes a vertical external gradient component that increases in the direction toward

the capture structure, and that the external gradient is high enough to transport magnetically-labeled material toward the capture structure. A magnetic field which could serve such dual purposes can be produced by various configurations of magnets. One advantageous arrangement of external source magnets is shown in FIG. 8.

[0056] FIG. 8 shows the separation vessel 10 positioned at a preferred location relative to a pair of opposed magnetic poles 23 and 24 having a gap formed therebetween. The lower surfaces of the poles 23 and 24 are tapered toward the gap, so that the magnetic field applied to the chamber is non-uniform, and has a substantially vertical gradient effective to urge magnetically-responsive particles within the chamber against the force of gravity toward the ferromagnetic collection structure on the upper wall.

[0057] FIG. 9 shows the paths that would be followed by such particles within the chamber in the absence of the ferromagnetic collection structure. As can be seen, the influence of the externally-applied gradient is sufficient to move the particles substantially vertically toward the upper wall of the chamber.

[0058] FIG. 10 shows the paths followed by particles within the chamber, including the effect thereon caused by the presence of a ferromagnetic collection structure comprising lithographically-defined lines having a thickness of 5.0 μm and a width of 20 μm . As can be seen, the externally applied gradient tends to urge particles initially located in the lower portion of the chamber to move into the high gradient regions generated by magnetization of the ferromagnetic lines.

[0059] The precise design parameters for the magnets 23 and 24 shown in FIG. 8 (and shown in connection with an automated observation system in FIG. 11) required to induce a desirably strong internal gradient and to apply a desirably strong vertical external gradient, will depend upon application-specific conditions such as the magnetization of the magnetic particles employed, the mass and size of the target entities, and the viscosity and temperature of the fluid medium. Those skilled in the art will be enabled hereby to select appropriate design parameters in view of such considerations. In experimental conditions such as are described herein, a pair of rare earth magnets (Crucible Magnets; Elizabethtown, Kentucky) having an internal magnetization of 1200 gauss and an acute taper angle of 20° separated by a distance of 5.0 mm to form a gap through which observations of collected entities could be made. The upper surface of the separation vessel was positioned 2.0 to 3.0 mm below the gap. Although Fig. 10 shows a chamber height of 200 μm , chamber heights of 1 mm have been modeled and actually used to collect and quantify magnetically labeled cells.

[0060] Of course, it is possible to exceed the capacity of the magnetic lines by attempting to collect so much target material, that a monolayer of target particles is no longer possible. In which case, the dilution of the test sample or the use of a larger chamber with a greater linear capacity of the magnetic lines is required.

[0061] Thus, analysis of the magnetically labeled target material is enabled. Alignment of the target material in a monolayer also allows for the analysis to be conducted by automated means, such as mechanical automated cell counting technology. The target material can be illuminated through the transparent wall member and the optical properties of the target material can be detected. Optical properties include the direct observation of the target material and the measurement of adsorbed, scattered or fluorescent light. Optionally, the target material can be analyzed aided by the addition of a substrate or other compound. Other compounds include the use of probes which recognize a characteristic determinant of the target material, and nuclear, cytoplasmic or membrane dyes. These probes can be either inherently fluorescent, fluorescently tagged, or fluorescent only upon binding to the characteristic determinant. Differentiation of the target material, i.e., leukocytes, is thus possible with a specific binding substance which recognizes subsets of the target material.

[0062] Although the above descriptions are exemplified by the collection of leukocytes, it is also possible to immobilize other types of cells in the apparatus of the invention. For example, platelets can be selected by use of CD41 or CD61. Differentiation into subsets includes an analysis of their activation status (recognition by CD62p or PAC-1) or the presence of granules (recognition by CD63 or LDS-751). The immobilization of red blood cells is discussed elsewhere in this specification.

[0063] A notable advantage of the apparatus and method of the invention is that the ability to provide linearly monolayered entities presents the opportunity to perform sequential reactions in a rapid and highly efficient manner. Thus, not only can the apparatus and method of the invention be used to facilitate cell analysis for the determination of cell surface characteristics, e.g., T-Cell, B-Cell Progenitor cells and subset markers thereof, but they can in principle be applied for the analysis of intracellular components or genes. Such an analysis would be done by first capturing and aligning the cells of interest. This step would be followed by a series of sequential flow-by reactions, which would permeate the cell membrane, tag entities of interest and amplify signal on tagged entities. This capability provides a distinct advantage over existing cytometric technologies.

[0064] The following examples further describe aspects of the present invention.

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EXAMPLE 3

[0065] A direct coated ferrofluid was prepared according to U.S. patent application 08/482,448. The ferrofluid parti-

cles were coated with CD45 antibody, which binds to leukocytes. The ferrofluid was stored in a HEPES buffer, pH 7.5 at 100 µg/ml.

[0066] The target cells were CEM cells at a concentration of 5×10^6 cell/ml. 100 µl of cells were incubated with 10-30 µl of ferrofluid for ten minutes at room temperature before loading the collection chamber.

5 [0067] A separation vessel was provided with a chamber having dimensions of 0.1 mm x 5 mm x 20 mm, for a chamber volume of 10 µl. The ferromagnetic capture structure comprised lithographically formed lines of nickel having dimensions of approximately 5 µm thick x 25 µm wide. The lines were spaced at 100 µm intervals.

10 [0068] After incubation, approximately 10 µl of the magnetically labeled CEM cells were loaded into the collection chamber. The interior of the chamber was first observed in the absence of a magnetic field and almost all of the cells were observed to have settled at the bottom of the chamber. The chamber was then agitated to resuspend the cells. Then, the collection chamber was placed into a magnetic field formed by two square magnets (such as the magnets shown in FIG. 2). The vessel was located in a non-uniform region of the field outside of the substantially uniform field directly between the magnets (i.e., at the location 33 shown in FIG. 6). Hence, an external gradient in the vertical direction was applied to the chamber to urge the magnetically responsive particles toward the ferromagnetic capture means.

15 [0069] While the collection chamber was positioned in the magnetic field, it was supported upon a microscope stage for observation of the cells were through a transparent wall of the chamber. Almost all of the target cells were observed to be aligned along the ferromagnetic capture lines in a single layer. Most of the cells were observed to be aligned after about ten seconds after the chamber was placed in the magnetic field. After a minute, all discernible cell movement ceased.

20 [0070] In another experiment, the loaded chamber was placed in a substantially homogenous magnetic field as indicated by the chamber 10 shown in Fig. 6. Although the magnetized wires collected approximately 50% of the magnetically labeled CEM cells, a large number of cells settled to the bottom of the collection chamber.

25 EXAMPLE 4

[0071] The experiment of Example 3 was repeated with human whole blood. 100 µl of whole blood was incubated with 10-30 µl of the CD45 direct labeled ferrofluid described in example 1. After a ten minute incubation at room temperature, the collection chamber was loaded with 10 µl of test sample and positioned in the magnet arrangement described above. A short settling period of one minute was required to allow the non-target red cells to settle to the bottom of the chamber and away from the ferromagnetic capture means to allow observation of the target leukocytes cells. Upon microscopic examination, the target cells were seen to be aligned along upon the ferromagnetic capture lines. In other experiments with fluorescently labeled nucleated cells, no settling period was required to distinctly identify the collected target cells (i.e. leukocytes).

30 [0072] Although this example illustrated the alignment of leukocytes in whole blood, it is possible to further distinguish or differentiate the leukocytes with the addition of probes to the desired subpopulations of leukocytes. As noted in example 1, it will be apparent to one skilled in the art that the method described in this example could be applied to numerous types of cell separation and/or differentiation.

40 EXAMPLE 5

[0073] The instant invention should also be useful for conducting competitive immunoassays. Proteins, hormones, or other blood components may be measured in whole blood using a device similar to that described in connection with FIGS. 1A and 1B. Magnetic particles which directly or indirectly bind to the blood components to be analyzed could be introduced into a blood sample, along with a fluorescent, chemiluminescent or other detectable probe, which binds directly or indirectly with the component to be analyzed. After the solution containing the blood, magnetically responsive particle, and detectable probe has been introduced into the device, the device is placed into a non-uniform magnetic field, and oriented such that gravity and the externally applied gradient act together to reinforce each other, instead of acting in opposition. The magnetically labeled protein, hormone, or other blood component will be drawn down to the magnetic collection structure. The excess detectable probe would remain in solution. The cellular components in the blood would also be drawn down towards the magnetic collection structure due to gravity. Detection of the non-bound detectable probe through fluorescence emission, chemiluminescence, or other means would be possible through a transparent wall of the collection device. The probe's signal, such as light emission, from the non-bound detectable probe would initially be blocked by cells, such red blood cells. Such cells would eventually settle to form a layer over the magnetic collection structure(s) to allow unobstructed detection of an emission fluorescence, or light scattering probe signal.

III. Automated Optical Analysis of Immobilized Target Entities

[0074] As noted above in connection with FIG. 6, the fringing field beneath a pair of opposed rectangular cross-section magnets is capable of providing the desired vertical external gradient while inducing internal magnetization of the ferromagnetic collection structure. For microscopic observation of the collected material, wherein the optical observation system is limited by a finite focal length such as less than 5mm, it is desirable to reduce the vertical distance between the top wall of the collection vessel and the top of the magnetic elements providing the field. In general, such an objective can be achieved in a magnetic arrangement having two opposing pole faces separated by a gap, wherein the pole faces are formed to have tapering surfaces toward the gap, such as shown in FIG. 8.

[0075] Providing a desirably short distance between the top of the magnetic arrangement and the top of the vessel permits the use of various automated observation means. Additionally, because target entities are collected in an orderly pattern on the interior surface of a transparent chamber, an automated observation system can be configured to provide relative motion between the vessel and the light gathering elements of the observation system in order to "track" the collected target entities for automated enumeration, which can include spectral analysis of light emitted or absorbed by the collected targets.

[0076] One such automated analysis system 100 is shown in FIG. 11. The analysis system 100 comprises optical tracking and beam analysis components similar to those employed for reading compact discs known in the audio and data storage arts. Briefly, a pair of laser diodes 110 and 112 are connected with a power supply 114 to generate respective parallel beams of light. One beam is employed by the analysis system for locating and tracking lines of the ferromagnetic collection structure. The other beam is used for detecting the presence of collected target entities adjacent to a located line. Relative motion between the optical elements of the analysis system 100 is provided by a mechanical tracking unit 116. Coordination of the functions of the analysis system 100 is provided by a microprocessor 118.

[0077] Laser 112 generates the tracking beam, which is transmitted through dichroic mirrors 120 and 122, and focussed upon the upper interior surface of the separation vessel 10 by objective lens 124. The tracking beam is reflected from the interior surface of the separation vessel, and is re-transmitted through dichroic mirrors 122 and 120 toward a photodetector 126. Photodetector 126 generates an electric signal in response to receiving the reflected light, which is provided to the microprocessor 118. The mechanical tracking unit 116 is operated by the microprocessor 118 to move the objective in the presumed direction of the lines of the ferromagnetic collection structure. Microprocessor 118 is programmed to detect deviations within the electrical signal from photodetector 126 to provide a feedback signal to the mechanical tracking unit 116 for adjusting the position of the objective 124 in a direction perpendicular to the lines of the ferromagnetic collection structure.

[0078] As the objective is moved to track the lines of the ferromagnetic collection structure, laser 110 is operated to generate a beam of light for detecting the presence of collected target entities. The light from laser 110 is transmitted through dichroic mirrors 128 and 122, and is focussed upon the upper interior surface of the collection vessel to form a spot adjacent to the focal point of the tracking beam. Light reflected by target entities will be transmitted through dichroic mirrors 122 and 128 toward photodetector 130. Photodetector 130 generates an electrical signal representative of the light reflected from the target entities, which is transmitted to microprocessor 118. Microprocessor 118 is programmed to monitor variations in the electrical signal from photodetector 130, in order to provide an analysis output signal, such as a counting signal at an output terminal 132. It will be appreciated that such an output signal can be further processed to provide information relative to the quantity and respective positions of the collected target entities.

[0079] In alternative embodiments, one laser could be used to illuminate the chamber, instead of two lasers as depicted in the analysis system 100. Optionally, the laser could be eliminated entirely and the chamber could be illuminated with a light-emitting diode or other light source, including light sources that illuminate the chamber from the sides or from below. In other embodiments, spectral analysis components, such as optical filters and gratings, as well as illuminating components having various spectral characteristics, can be employed in an automated analysis system for conducting spectral analysis of light emitted from the collected entities as an objective lens of the analysis system is moved to track the ferromagnetic collection structure.

[0080] In some cases, it may be desirable to include means to vibrate the chamber, to prevent magnetic particles from being held by friction against the walls of the chamber. Vibrating the chamber has been found to increase magnetic separation efficiency under such circumstances. To facilitate vibration, the separation vessel may be mounted on a piezoelectric crystal 123 connected to an electric power source for vibrating the chamber at a desired frequency.

IV. Quantitative Determinations of Biological Fluid ComponentsA. Rare Species Enrichment and Sample Preparation

[0081] With decreasing frequency of a target population it becomes increasingly more difficult to reliably detect, enumerate and examine the target population. Not only is there an increasing demand on the specificity of the identifiers,

i.e., probes or a combination of probes, but the need arises for a specific target enrichment technique in addition to the need to process larger volumes of the bodily fluid. Table II below illustrates this by showing the frequencies of various cell populations among the nucleated cells in peripheral blood of normal individuals.

5

TABLE II

10

15

| Cell Frequency | Cell Number | Targets Cells |
|--------------------------------------|-----------------------|---------------------------|
| 1:1-1:10 | 10,000-1,000/ μ l | granulocytes, lymphocytes |
| 1:10-1:10 ² | 1,000-100/ μ l | monocytes, eosinophils |
| 1:10 ² -1:10 ³ | 100-10/ μ l | basophils |
| 1:10 ³ -1:10 ⁴ | 10-1/ μ l | CD34+ cells |
| 1:10 ⁴ -1:10 ⁵ | 1,000-100/ml | CD34+, CD38- cells |
| 1:10 ⁵ -1:10 ⁶ | 100-10/ml | tumor cells |
| 1:10 ⁶ -1:10 ⁷ | 10-1/ml | tumor cells |
| 1:10 ⁷ -1:10 ⁸ | 1,000-100/l | tumor cells |

[0082] For analysis of infrequent cells, such as CD34+ cells or a subset thereof, or in case of disease potential circulating tumor cells, the amount of blood needed to reliably detect, enumerate and examine the target population needs to be substantially larger than 1 μ l. One practical implication for the analysis of larger blood volumes is a substantially longer processing time. For example, for flow cytometric analysis of a 1 ml blood sample the erythrocytes in the sample are typically lysed, which is accompanied with a 10 fold dilution of the sample. For a typical sample flow rate of 1 μ l/sec, the 10 ml volume of the lysed sample will thus require 2.78 hours for analysis. The need for enrichment of the target population and an increase in its concentration is thus clearly desired. A variety of enrichment methods can be employed to increase the concentration of the desired target in the sample to be analyzed, so that a sufficient number of target entities will be present in the separation vessel to permit detection. Success of these procedures is determined by carry over of non targets, recovery of targets, the ability to concentrate the target and the ability to accurately analyze the target after the procedure. Introduction into a separation vessel of a sample from a bodily fluid of which targets are concentrated and non targets are reduced, permits enumeration and examination of the target population.

[0083] In one method of sample preparation, an external gradient separator of the type described in U.S. Patent No. 5,186,827 may be employed. For example, to a vessel containing 10 ml of blood, a bioactive ferrofluid is added which identifies cells of epithelial origin. After appropriate incubation, the sample is placed in the external gradient separator. After separation, the blood components not magnetically attached to the wall of the vessel are removed while the desired target substance remains adhered to the wall of the separator. The separated cells can now be resuspended into a smaller volume when the separation vessel is removed from the magnetic field.

[0084] To the resuspended sample, fluorescent labeled probes can be added. After incubation, the sample is again placed in an external gradient magnetic separator. After separation, the supernatant (including excess reagents) is removed, and the separated cells are resuspended in a volume commensurate with the chamber of a separation vessel of the present invention. Assuming the 10 ml of blood contained 10⁸ cells, a carryover of 0.01% would result in 10⁴ cells, which is within the range of the cell capture capacity of the apparatus of this invention. Given a target cell frequency of 1 in 10⁷ and a capture efficiency of 70%, 7 target cells would be captured, which is sufficient for identification and further characterization. The cells which express the antigen targeted by the ferrofluid will align along the ferromagnetic lines in addition to other cells which are nonspecifically bound to the ferrofluid. Cells which were captured due to other reasons, such as entrapment, will not align, resulting in a further purification of infrequent cell types.

[0085] Identification and further characterization of the target cells can be obtained by the differences in the scattered and spectrum of the fluorescence light. An additional improvement can be achieved by utilization of a fluorescent form of the bioactive ferrofluid, the target cells can then be discriminated from non specifically bound cells by the amount of fluorescence emitted by the cells, i.e. the density of the antigen on the cell surface of the target cell is most likely different from the density of nonspecifically bound ferrofluid to non target cells. In contrast with flowcytometry the individual targets isolated by means of the present invention can be reexamined, in that an optical detection system can be configured to identify and record the location of the target particles of interest. Once the location of the target cells has been detected and recorded, the immobilization vessel then can be examined by a more sophisticated optical detection system, such as a confocal microscope.

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B. Quantitative Analysis

[0086] For conducting quantitative analysis of certain cell types, a difficulty arises from having to perform multiple

dilutions of the original blood sample prior to magnetically capturing the cell type of interest. After having performed multiple dilutions, determining the concentration of the captured species relative to the original blood volume requires knowledge of the precise dilution ratio and the magnetic capture efficiency. These quantities can be determined by adding concentration markers to the original blood sample.

- 5 [0087] A first marker, for determining the dilution, comprises a known concentration of distinctly identifiable particles that are loaded with sufficient magnetically responsive material to be captured with substantially total efficiency. The second marker, for determining the magnetic capture efficiency of the target cells, comprises a known concentration of distinctly identifiable particles that are loaded with approximately the same quantity of magnetically-responsive material as the target cell. The second marker can comprise magnetically responsive beads that have been formed with 10 sufficient magnetic material to have a magnetic moment substantially equal to that of the ferrofluid-labeled target entities, and similar fluid transport behavior. Alternatively, the second marker may comprise magnetically inert bodies that are coated with a binding substance having substantially the same number of binding sites and binding affinity as the target cells. Other techniques can be used to provide the second marker with analogous collection behavior relative to the target entity. Such methods are discussed in the following examples.

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EXAMPLE 6

Concentration Calibrated Sample Preparation Using Magnetically-Loaded Markers

- 20 [0088] To 10 ml of blood are added 5 ml of reagent containing an epithelial cell specific ferrofluid, 10,000 green fluorescent 10 µm beads with approximately 500 ferrofluid particles per bead affixed thereto and 10,000 red fluorescent 10 µm beads with approximately 5,000 ferrofluid particles per bead affixed thereto. After 15 minutes of incubation, the sample is placed in a magnetic separator of the type described in U.S. Patent No. 5,186,827 for 10 minutes. That portion of the sample not attached to the wall of the vessel is discarded, the vessel is removed from the magnetic field 25 and the cells collected on the wall are resuspended in 2 ml of a solution, such as an isotonic buffer or a solution which permeabilizes the cell membrane. The resuspended cells are placed in the magnetic field for 5 minutes and the sample not attached to the wall of the vessel is discarded. The cells collected on the wall are resuspended in 0.2 ml of a solution containing fluorescently labeled antibodies, for example, CD45 PerCP identifying leukocytes, anti EPCAM PE and/or 30 anticytokeratin PE identifying epithelial cells. Optionally, the sample may again be separated, excess antibodies discarded and the collected cells resuspended in a solution containing a nucleic acid dye with fluorescence properties which can be spectrally distinguished from the fluorescence produced by the fluorescent conjugated monoclonal antibodies.

- [0089] Leukocytes, epithelial cells, green and red beads can then be enumerated by the methods described herein or by traditional enumeration methods. A measurement of, for example, 10 epithelial cells, 5000 green beads and 7000 red beads would indicate that in case the epithelial cells have a density of 500 ferrofluid particles/cell their concentration would be $(10 \times 10,000/5000)/10=2$ epithelial cells per ml of blood; and in case the epithelial cells have a density of 5,000 ferrofluid particles / cell their concentration would be $(10 \times 10,000/7,000)/10=1.4$ epithelial cells per ml of blood.

- [0090] Although this example describes the use of two makers to accurately determine epithelial cell concentration, it will be apparent that any cell concentration can be determined.

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EXAMPLE 7

Sample Preparation Using Ferrofluid-Binding Markers

- 45 [0091] The cell analysis of Example 6 was repeated, except for the addition of 10,000 red and 10,000 green beads with respectively 500 and 5,000 antigens per bead to the blood. (Many of the antigens are cloned and recombinant proteins can be obtained which are recognized by the antibodies). This is followed by addition of the ferrofluid which identifies both beads and epithelial cells. In this example, an accurate estimate of the absolute number of target cells is obtained and it determines in addition whether the target cell specific ferrofluid works.

- 50 [0092] Examples 6 and 7, above, describe a sample preparation procedure to control for performance of a cell analysis system, as well as indicate the concentration of the measured target cells per volume unit. An analysis sample prepared according to the procedure described above can then be quantitatively analyzed using flow cytometry, or with the apparatus described herein. The volume in the apparatus described herein is known, whereas the volume which passes through a flow cytometer has to be determined by the beads or by the actual measurement of the volume 55 in which the flow cytometer measured the target events. Using precise sample dispensing techniques (pipette) the volume of the sample, the reagent and the diluent is accurately measured. In the comparable procedure using the apparatus of the present invention, on the other hand, accurate dispensing of sample and reagents is sufficient to determine the cell concentration (without also requiring a count of the beads for volume determination). In its simplest

configuration, however, it is desired to obviate precise dispensing of sample. To this end, the bead approach described above can be used to determine the precise dilution of the sample, rather than the determination of the precise volume from which the target cells are analyzed, as exemplified above.

5 **Example 8**

Cell Concentration Determination Using Calibrated Marker Solution

[0093] Approximately 50 µl of a solution containing target cell specific ferrofluid, reagents such as fluorescent nucleic acid dyes to facilitate identification of target cells and a known concentration of 10 µm beads, for example, 1,000 per µl, with physical properties which distinguish them from the target cells, and labeled with an amount of ferrofluid which is in the same range as that of the target cells, are introduced into a separation vessel of the present invention. A drop of blood is also introduced into the vessel by, for example, capillary action. The blood and fluid are mixed and incubated. The vessel is now introduced into a magnetic field and the target cells and beads are aligned along the ferromagnetic lines. From the number of beads counted, the actual volume of blood which was mixed with the fluid can now be determined. For example, if 6,000 beads are measured and the chamber volume is 10 µl, the volume of the marker fluid in the chamber is $6,000/1,000=6$ µl. The blood volume in the chamber is thus $10 \mu\text{l} - 6 \mu\text{l} = 4 \mu\text{l}$. If 32,000 target cells are measured, the target cell concentration is $32,000/4 = 8,000$ cells/µl. As should be evident from the foregoing description, neither the exact volume of the blood nor the exact volume of the marker fluid has to be known so long as the concentration of the beads in the marker fluid is known and the blood and bead solution are fully mixed.

[0094] As in Example 7, beads can be added which have an amount of antigen which is similar to the amount of antigen on the target cells. In addition to the features described in examples 5, 6, and 7, this procedure further provides a method to determine the efficacy of the techniques and reagents used to select and detect the target material.

25 **C. Assessment of Red Blood Cell Parameters**

[0095] Important parameters in hematology are hemoglobin and hematocrit, mean corpuscular volume (MCV), mean hemoglobin concentration (MCH), mean cellular hemoglobin concentration (MCHC) and red blood cell number (RBC). To measure erythrocytes, a larger dilution of the blood is required as compared to leukocyte measurements or subsets thereof (on the order of a 1,000 fold higher concentration). A higher fluid volume can be used in the chamber in order to obtain a greater dilution. It also could be advantageous to reduce the chamber height to reduce the number of erythrocytes aligned. Erythrocytes can be identified and aligned by using an erythrocyte specific ferrofluid such as glycophorin A-labeled ferrofluid or transferrin-labeled ferrofluid, the latter recognizing only the immature reticulocytes, i.e., RNA containing erythrocytes. Erythrocytes bound to such ferrofluids can be distinguished from nucleated cells bearing the transferrin receptor by the absence of nuclear fluorescence. Alternatively, one could make use of the presence of hemoglobin in the erythrocytes to render the erythrocytes magnetically responsive. The iron present in hemoglobin can be reduced, or otherwise rendered magnetic according to a known procedure such that the red blood cells will be immobilizable by the internal gradients generated in the vicinity of the ferromagnetic lines. In such a method, no ferrofluid is necessary to attract the erythrocytes to the ferromagnetic lines, and the rate at which they would be attracted toward the ferromagnetic lines would be proportional to the amount of hemoglobin in the cells.

[0096] Once the erythrocytes are aligned along the ferromagnetic lines, light scatter and absorption measurements of the individual cells can be performed which permit the assessment of size, volume and hemoglobin content according to procedures for conducting measurements in known hematology analyzers, and suitably adapted for use with the present apparatus.

[0097] The ability to assess the shape of the individual erythrocytes provides clinically useful information. Elliptocyte, leptocyte, teardrop rbc, spherocytes, sickle cells, schistocyte, acanthocyte, echinocyte, stomatocyte, xerocyte are all red blood cell shapes associated with disease states which cannot be accurately defined by the assessment of erythrocytes in hematology analyzers. Optionally, the erythrocytes can be further differentiated by the addition of probes which recognize erythrocytes subsets, such as CD71 or transferrin. The fluorescence or other detectable signal of the probe could thus be analyzed through the transparent wall member. Enhancement of the ability to assess red blood cell shapes could be significantly improved by measurement of the surface area of the cell membrane which can be achieved by the addition of a fluorescent membrane dye to the diluent fluid. Measurement of the total amount of fluorescence per cell is then proportional to the total amount of the membrane. From the surface area of the cell membrane and the analysis of the light scatter and fluorescence signals, the shape of the erythrocyte can be derived. The dilution of the blood sample can be determined by the addition of the beads to the diluent fluid, and the number and volume of the erythrocytes can be determined, the hematocrit (volume of cells/(total blood volume)) can also be determined according to the present method.

EXAMPLE 9**Measurement of Immune Status and Function in Whole Blood**

- 5 [0098] One of the critical needs for the immune function is the ability of lymphocytes to re-circulate through the various tissues of the body. This circulation uses blood and lymph for transportation and the antigen receptors present on the lymphocyte surface enable the monitoring of any site where antigen could enter the bloodstream. Memory T cells specific for antigens which are associated with specific infectious diseases such as measles, mumps, tetanus, HIV or Lyme disease are infrequent. However, their number increases dramatically when antigen reenters the body.
- 10 10 Monoclonal antibodies specific for disease specific T cells can be made and upon labeling with ferrofluid the antigen (disease)-specific Memory T cells can be aligned and enumerated. Alternately, all specific or subsets of T cells could be aligned. The apparatus of the current invention could then be used to determine specific responses of cell subsets to immunological stimuli.
- [0099] Sequential flow-by reactions of generic or disease specific antigens or other markers also could be used to
- 15 determine the functional status and capability among other characteristics of the aligned cells. A particular advantage of the current invention is that the reaction of individual cells can be measured at various time points after stimulation. This type of analysis is an obvious advantage over the measurement of immune function by flowcytometry which permits only one opportunity to measure individual cell responses.
- [0100] An arrangement for conducting "flow-by" or sequential reactions upon immobilized target material is shown
- 20 in FIG. 12. Fluid supply means, such as pumps, gravity reservoirs or syringes 220, 222 are provided for containing the test sample and respective reagents for reaction with the target material. Fluid conduits 224, 226 are provided for conducting flows of the respective fluids to a mixing valve 228. The mixing valve 228 is configured to select one or more of the fluid components for mixing and delivery to an inlet port 230 of the separation vessel 210. The separation vessel 210 further includes an outlet port 232 for conducting a flow of fluid out of the separation vessel 210. The
- 25 separation vessel 210 is configured for use with magnetic arrangements and manual or automatic optical observation means such as have been described above, so that target material may be immobilized therein by a ferromagnetic capture structure 218, and then subjected to sequential reactions with the fluids provided by the fluid supply means and selected by the multi-port valve 228 in sequence and/or combination.
- [0101] In a particularly preferred embodiment, the apparatus in FIG. 12 can be utilized to perform fluorescence *in situ* hybridization in order to differentiate among characteristics of cell subpopulations. In such a method, the desired types of cells are immobilized in the apparatus. The cell membranes are permeabilized by an appropriate reagent prior to, or after, immobilization. Then, *in situ* hybridization is performed on the immobilized, permeabilized cells by conducting sequential flows of reagents through the vessel. For fluorescent hybridization, the respective reagents comprise
- 30 respective fluorescent probes.
- [0102] The instant invention is particularly well adapted for performing sequential reactions due to the small size of the ferromagnetic lines relative to other internal gradient separators. The lines can be made relatively thin, due to the mechanical support provided by the wall of the vessel. Consequently, cells or other entities are rendered strongly immobilized by the relatively large internal gradients generated in the vicinity of the lines. Such strong immobilization force results in a greater resistance to hydraulic forces incident to conducting flow-by reactions on immobilized substances. Heretofore, magnetic capture structures were of significantly larger dimensions in order to provide an internal gradient with sufficient spatial extent to draw magnetic particles into immobilization. In accordance with the present invention, it has been found that gravitational force and/or an applied external gradient can be employed to draw magnetizable entities toward a strong internal gradient region generated in the vicinity of a capture structure having
- 35 relatively small dimensions, and supported along one side of the chamber of the separation vessel.
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Claims

1. An apparatus for observing magnetically-responsive microscopic entities suspended in a fluid medium, comprising:
- 50 a vessel having a transparent wall and a chamber formed therein for containing the fluid medium; a ferromagnetic capture structure formed on the transparent wall by means of a lithographic process; and magnetic means for inducing an internal magnetic gradient in the vicinity of the ferromagnetic capture structure, whereby the magnetically-responsive entities are immobilized along the wall adjacent to the capture structure.
- 55
2. The apparatus of claim 1 wherein the vessel comprises a fluid inlet and a fluid outlet for conducting a flow of fluid through the vessel.

3. The apparatus of claim 2 comprising fluid supply means connected with the fluid inlet for sequentially supplying a plurality of reagents to the fluid inlet.
5. The apparatus of any of claims 1 to 3 wherein the ferromagnetic capture structure comprises a plurality of ferromagnetic lines having an effective diameter of less than 30 µm.
10. The apparatus of any of claims 1 to 4 wherein the magnetic means is configured to apply an external gradient to the vessel for transporting entities within the vessel in the direction of the capture structure.
15. The apparatus of any of claims 1 to 5, comprising an automated optical observation system for detecting and enumerating the immobilized entities.
20. The apparatus of claim 6 wherein the automated observation system comprises:
 - an optical detector for detecting said immobilized entities; and tracking means for scanning the optical detector parallel to the ferromagnetic lines.
25. An apparatus for observing magnetically-responsive microscopic entities suspended in a fluid medium, comprising:
 - a vessel having a transparent wall and a chamber formed therein for containing the fluid medium;
 - a ferromagnetic capture structure supported on the transparent wall;
 - magnetic means for inducing an internal magnetic gradient in the vicinity of the ferromagnetic capture structure, whereby the magnetically-responsive entities are immobilized along the wall adjacent to the capture structure; and
 - an automated optical observation system for detecting and enumerating the immobilized entities.
30. The apparatus of claim 8 wherein the automated observation system comprises:
 - an optical detector for detecting said immobilized entities; and optical tracking means for scanning the optical detector along the capture structure.
35. The apparatus of claim 8 wherein the automated observation system comprises a vibratable support for supporting and selectively vibrating the vessel.
40. The apparatus of claim 10 wherein the vibratable support comprises a piezoelectric vibrator.
45. An apparatus for observing magnetically-responsive microscopic entities suspended in a fluid medium, comprising:
 - a vessel having a transparent wall and a chamber formed therein for containing the fluid medium;
 - a ferromagnetic capture structure supported on the transparent wall;
 - magnetic means for inducing an internal magnetic gradient in the vicinity of the ferromagnetic capture structure, and for applying an external gradient perpendicular to the ferromagnetic capture structure, whereby the magnetically-responsive entities are transported toward and immobilized along the wall adjacent to the capture structure.
50. The apparatus of claim 12 wherein the magnetic means comprises a pair of magnets having tapered pole surfaces positioned to provide a gap therebetween for receiving the vessel.
55. The apparatus of claim 12 or 13 wherein the ferromagnetic capture structure comprises a plurality of ferromagnetic lines having an effective diameter of less than 30 µm.
15. The apparatus of claim 14 wherein the ferromagnetic lines are supported on the wall by adhesion.
16. The apparatus of claim 15 wherein the ferromagnetic lines are formed on the wall by means of a lithographic process.
17. A method of immobilizing microscopic magnetically-responsive entities suspended in a fluid medium for observation, comprising the steps of:

- providing a vessel having a chamber with a transparent wall and a ferromagnetic capture structure supported on the wall;
introducing the fluid medium into the chamber;
applying a magnetic field to the chamber to induce an internal magnetic gradient in the vicinity of the ferromagnetic capture structure; and
observing the magnetically responsive entities held against the wall adjacent to the ferromagnetic capture structure.
- 5
18. The method of claim 17 wherein the ferromagnetic capture structure comprises a plurality of linear ferromagnetic elements, and said observing step comprises the step of scanning along the linear ferromagnetic elements with an automated optical detection system.
- 10
19. The method of claim 17 wherein said observing step comprises the step of enumerating the magnetically responsive entities.
- 15
20. The method of any of claims 17 to 19 wherein said applying step comprises the step of orienting the vessel to position the ferromagnetic capture structure beneath the chamber such that the magnetically responsive entities are transported toward the capture structure under the influence of gravity.
- 20
21. The method of any of claims 17 to 19 wherein the applying step comprises applying an external magnetic gradient to the vessel to transport the magnetically-responsive entities toward the capture structure.
- 25
22. A method of immobilizing microscopic magnetically-responsive entities suspended in a fluid medium for observation, comprising the steps of:
- providing a vessel having a chamber with a transparent wall and a ferromagnetic capture structure supported on the wall;
introducing the fluid medium into the chamber;
applying a magnetic field having an external gradient to the chamber to induce an internal magnetic gradient in the vicinity of the ferromagnetic capture structure and to transport magnetically-responsive entities toward the capture structure;
collecting said magnetically-responsive entities in a linear monolayer against the wall and adjacent to the ferromagnetic capture structure; and
observing the magnetically responsive entities held against the wall adjacent to the ferromagnetic capture structure.
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23. A method of differentiating biological substances of at least a first and second type in a fluid sample, comprising the steps of:
- marking the first and second types of biological substances with respective markers to produce distinctive optical characteristics;
adding to the fluid sample a ferrofluid having a binding affinity for biological substances of the first and second types;
placing the fluid sample in a separation vessel having a transparent wall defining one side of a chamber, and having a ferromagnetic capture structure supported along the wall;
positioning the vessel in a magnetic field for inducing an internal gradient in the vicinity of the capture structure sufficient to immobilize the biological substances of the first and second types along the wall adjacent to the capture structure; and
observing the distinct optical characteristics of the respective first and second types of the biological substance.
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24. The method of claim 23 wherein said marking step comprises:
- adding to the fluid sample first and second optical markers adapted for producing distinct optical responses from biological substances of a respective first type and a second type;
and wherein said observing step comprises the step of illuminating the vessel with optical radiation adapted to produce said distinct optical responses.
- 55
25. The method of claim 23 or 24 wherein the first and second biological substances are cell types of respective

leukocyte sub-populations.

26. The method of claim 25 wherein the leukocyte sub-populations comprise respective living and dead leukocytes.

5 27. The method of claim 23 or 24 wherein the first and second biological substances are respective first and second red blood cell subpopulations.

28. The method of claim 23 or 24 wherein the first and second biological substances are cell types of respective platelet
10 sub-populations.

10 29. The method of claim 23 wherein said observing step comprises observing distinct shapes of the respective first and second types of the biological substances.

15 30. The method of claim 23 wherein said biological substances are cell types, and said observing step comprises enumerating the immobilized cells of the first and second types and determining a ratio of immobilized substances relative to the net surface area of the immobilized cells.

31. The method of claim 23, comprising the steps of:

20 diluting the fluid sample with a diluent containing a fluorescent membrane dye; and

wherein said observing step comprises measuring fluorescence from the immobilized cells to determine the proportionate surface area of the immobilized cells.

25 32. The method of any of claims 23 to 31 comprising the step of conducting sequential flows of reagents through the vessel to produce the distinct optical responses.

33. The method of claim 32 wherein the biological substances are respective cells of distinct cell subpopulations, the method comprising the step of permeabilizing the membranes of the cells prior to conducting the sequential flows
30 of reagents through the vessel.

34. The method of claim 32 or 33, wherein the sequential flows of reagents comprise sequential flows of reagents for producing distinct fluorescent optical responses, and wherein said observing step comprises illuminating the immobilized cells with illumination selected to produce said distinct fluorescent responses.

35 35. The method of claim 23 wherein said biological substance comprises cells and said observing step comprises conducting in situ hybridization of the immobilized cells.

40 36. A method of identifying immunophenotypic sub-populations of biological substances suspended in a fluid medium, comprising the steps of:

adding to the fluid medium a quantity of magnetically-responsive particles having a binding affinity for the biological substances;

adding to the fluid medium a plurality of probes having respective distinct fluorochromes;

45 placing the fluid medium in a separation vessel having a transparent wall defining one side of a chamber, and having a ferromagnetic capture structure supported along the wall;

positioning the vessel in a magnetic field for inducing an internal gradient in the vicinity of the capture structure sufficient to immobilize the biological substances along the wall adjacent to the capture structure;

illuminating the vessel with optical radiation adapted to excite at least a selected one of the fluorochromes; and
50 observing the optical response of the selected fluorochrome.

37. A method of differentiating red blood cell subpopulations in a fluid sample, comprising the steps of:

rendering red blood cells in the fluid sample to be magnetically responsive;

55 placing the fluid sample in a separation vessel having a transparent wall defining one side of a chamber, and having a ferromagnetic capture structure supported along the wall;

positioning the vessel in a magnetic field for magnetizing the magnetically responsive red blood cells and for inducing an internal gradient in the vicinity of the capture structure sufficient to attract and immobilize the cells

- along the wall adjacent to the capture structure; and
observing red blood cells immobilized adjacent the capture structure.
- 5 38. The method of claim 37, wherein the rendering step comprises the step of labelling the red blood cells with magnetizable particles.
39. The method of claim 38 wherein the magnetizable particles are adapted to bind with a characteristic determinant of at least one subpopulation of red blood cells.
- 10 40. The method of claim 39 wherein the magnetizable particles comprise at least one binding substance selected from the group consisting of CD71, glycophorin A and transferrin.
- 15 41. The method of any of claims 37 to 40, wherein the observing step comprises enumerating red blood cell subpopulations having respective distinct shapes.
- 15 42. The method of any of claims 37 to 41, comprising the steps of:
 diluting the fluid sample with a diluent containing a fluorescent membrane dye; and
 20 wherein said observing step comprises measuring fluorescence from the immobilized cells to determine the proportionate surface area of the immobilized cells.
43. A method of performing quantitative analysis of cells in a first fluid medium, comprising the steps of:
 25 adding to the first fluid medium a first marker comprising a known quantity of first magnetically responsive particles;
 adding to the first fluid medium a quantity of second magnetically responsive particles having a binding affinity for the cells;
 30 placing the first fluid medium in a magnetic separator for separating magnetically-responsive entities from other components of the first fluid medium;
 resuspending the separated magnetically-responsive entities in a second fluid medium;
 enumerating the quantity of the first marker and the cells in the second fluid medium to determine the concentration of the cells in the first fluid medium.
- 35 44. The method of claim 43, comprising the steps of:
 placing the second fluid medium into a chamber of a separation vessel having a transparent wall and a ferromagnetic capture structure supported along the wall;
 positioning the separation vessel in a magnetic field to generate an internal magnetic gradient in the vicinity of the ferromagnetic capture structure; and
 40 enumerating cells immobilized against the transparent wall adjacent to the ferromagnetic capture structure.
44. The method of claim 43, comprising the steps of:
 45 adding to the first fluid medium a second marker comprising a known quantity of third magnetically responsive particles having a magnetic moment approximately equal to the bound cells; and
 wherein said enumerating step comprises the step of enumerating the quantity of the second marker in the second fluid medium as a measurement of the magnetic separation efficiency.
- 50 45. The method of claim 43 or 44, comprising the step of preparing the second marker by providing a quantity of non-magnetically-responsive particles bearing a binding agent for the second magnetically responsive particles.
- 55 46. The method of claim 43 wherein said enumerating step comprises the step of providing the second fluid medium to a flow cytometer.
47. A method of measuring a hematological parameter of a fluid sample containing red blood cells, comprising:

5 diluting a first fluid sample comprising blood with a diluent containing a known quantity of first magnetic particles, to obtain a second fluid sample;
 rendering red blood cells one of the first and second fluid samples to be magnetically responsive;
 placing the second fluid sample into a chamber of a separation vessel having a transparent wall and a ferromagnetic capture structure supported along the wall;
 positioning the separation vessel in a magnetic field for magnetizing the red blood cells and for generating an internal magnetic gradient in the vicinity of the ferromagnetic capture structure; and
 10 enumerating red blood cells and first magnetic particles immobilized against the transparent wall adjacent to the ferromagnetic capture structure.

- 10 48. The method of claim 47, wherein the rendering step comprises the step of enhancing magnetizability of the red blood cells by adding to one of the first and second fluid samples a second magnetic particle having a binding affinity for red blood cells.
- 15 49. The method of claim 47 or 48, comprising the step of adding to one of the first and second fluid samples a known quantity of a third magnetic particle having a magnetic characteristic comparable to the red blood cells rendered magnetically responsive; and wherein said enumerating step comprises enumerating third magnetic particles immobilized adjacent the capture structure.
- 20 50. The method of any of claims 47 to 49, comprising the step of determining, on the basis of said enumeration, at least one of the parameters of hemoglobin content, hematocrit, mean corpuscular volume, mean hemoglobin concentration, and red blood cell number.

25 **Patentansprüche**

1. Gerät zur Beobachtung magnetisch reagierender mikroskopischer Teilchen, die in einem flüssigen Medium suspendiert sind, umfassend: ein Gefäß mit einer durchsichtigen Wand und einer darin geformten Kammer zur Aufnahme des flüssigen Mediums; eine ferromagnetische Fangkonstruktion, die in einem lithografischen Verfahren auf der durchsichtigen Wand geformt wird; und ein magnetisches Mittel zur Induzierung eines internen magnetischen Gefäßes in der Nähe der ferromagnetischen Fangkonstruktion, wodurch die magnetisch reagierenden Teilchen über die Wand neben der Fangkonstruktion immobilisiert werden.
2. Gerät nach Anspruch 1, **dadurch gekennzeichnet, dass** das Gefäß einen Flüssigkeitseinlass und einen Flüssigkeitsauslass zur Durchleitung einer Flüssigkeitsströmung durch das Gefäß umfasst.
3. Gerät nach Anspruch 2, umfassend ein mit dem Flüssigkeitseinlass zur sequentiellen Zufuhr mehrerer Reagenzien zum Flüssigkeitseinlass verbundenes Flüssigkeitzzuführmittel.
4. Gerät nach einem der Ansprüche 1 bis 3, **dadurch gekennzeichnet, dass** die ferromagnetische Fangkonstruktion mehrere ferromagnetische Linien mit einem wirksamen Durchmesser unter 30 µm umfasst.
5. Gerät nach einem der Ansprüche 1 bis 4, **dadurch gekennzeichnet, dass** das magnetische Mittel so ausgestaltet ist, dass es zum Transport von Teilchen im Gefäß in Richtung der Fangkonstruktion ein externes Gefäß an das Gefäß anlegt.
6. Gerät nach einem der Ansprüche 1 bis 5, umfassend ein automatisches optisches Beobachtungssystem zum Nachweis und Zählen der immobilisierten Teilchen.
7. Gerät nach Anspruch 6, **dadurch gekennzeichnet, dass** das automatische Beobachtungssystem Folgendes umfasst: einen optischen Detektor zum Nachweis der immobilisierten Teilchen; und Verfolgungsmittel zum Scannen des optischen Detektors parallel zu den ferromagnetischen Linien.
8. Gerät zum Beobachten magnetisch reagierender mikroskopischer Teilchen, die in einem flüssigen Medium suspendiert sind, umfassend: ein Gefäß mit einer durchsichtigen Wand und einer darin geformten Kammer zur Aufnahme des flüssigen Mediums; eine ferromagnetische Fangkonstruktion, die auf der durchsichtigen Wand festgehalten wird; ein magnetisches Mittel zur Induzierung eines internen magnetischen Gefäßes in der Nähe der ferromagnetischen Fangkonstruktion, wodurch die magnetisch reagierenden Teilchen über die Wand neben der Fang-

- konstruktions immobilisiert werden; und ein automatisches optisches Beobachtungssystem zum Nachweis und Zählen der immobilisierten Teilchen.
9. Gerät nach Anspruch 8, **dadurch gekennzeichnet, dass** das automatische Beobachtungssystem folgendes umfasst: einen optischen Detektor zum Nachweis der immobilisierten Teilchen; und optische Verfolgungsmittel zum Scannen des optischen Detektors entlang der Fangkonstruktion.
 10. Gerät nach Anspruch 8, **dadurch gekennzeichnet, dass** das automatische Beobachtungssystem einen vibrationsfähigen Träger zum Unterstützen und selektiven Vibrieren des Gefäßes umfasst.
 11. Gerät nach Anspruch 10, **dadurch gekennzeichnet, dass** der vibrationsfähige Träger einen piezoelektrischen Vibrator umfasst.
 12. Gerät zum Beobachten magnetisch reagierender mikroskopischer Teilchen, die in einem flüssigen Medium suspendiert sind, umfassend: ein Gefäß mit einer durchsichtigen Wand und einer darin geformten Kammer zur Aufnahme des flüssigen Mediums; eine ferromagnetische Fangkonstruktion, die auf der durchsichtigen Wand festgehalten wird; ein magnetisches Mittel zur Induzierung eines internen magnetischen Gefälles in der Nähe der ferromagnetischen Fangkonstruktion und zum Anlegen eines externen Gefälles lotrecht zur ferromagnetischen Fangkonstruktion, wodurch die magnetisch reagierenden Teilchen zur Wand neben der Fangkonstruktion transportiert und darauf immobilisiert werden.
 13. Gerät nach Anspruch 12, **dadurch gekennzeichnet, dass** das magnetische Mittel ein Magnetpaar mit spitz zulaufenden Polflächen umfasst, die so angeordnet sind, dass dazwischen zur Aufnahme des Gefäßes eine Lücke entsteht.
 14. Gerät nach Anspruch 12-13, **dadurch gekennzeichnet, dass** die ferromagnetische Fangkonstruktion mehrere ferromagnetische Linien mit einem wirksamen Durchmesser unter 30 pm umfasst.
 15. Gerät nach Anspruch 14, **dadurch gekennzeichnet, dass** die ferromagnetischen Linien durch Adhäsion an der Wand gehalten werden.
 16. Gerät nach Anspruch 15, **dadurch gekennzeichnet, dass** die ferromagnetischen Linien durch ein lithografisches Verfahren auf der Wand geformt werden.
 17. Verfahren zur Immobilisierung mikroskopischer, magnetisch reagierender Teilchen, die in einem flüssigen Medium suspendiert sind, für Beobachtungszwecke, umfassend folgende Schritte: Bereitstellen eines Gefäßes mit einer Kammer mit einer durchsichtigen Wand und einer ferromagnetischen Fangkonstruktion, die auf der Wand festgehalten wird; Einführen des flüssigen Mediums in die Kammer; Anlegen eines Magnetfelds an die Kammer, um ein internes magnetisches Gefälle in der Nähe der ferromagnetischen Fangkonstruktion zu induzieren; und Beobachten der magnetisch reagierenden Teilchen, die neben der ferromagnetischen Fangkonstruktion gegen die Wand gehalten werden.
 18. Verfahren nach Anspruch 17, **dadurch gekennzeichnet, dass** die ferromagnetische Fangkonstruktion mehrere lineare ferromagnetische Elemente umfasst und der Beobachtungsschritt den Schritt des Scannens entlang den linearen ferromagnetischen Elementen mit einem automatischen optischen Nachweissystem umfasst.
 19. Verfahren nach Anspruch 17, **dadurch gekennzeichnet, dass** der Beobachtungsschritt den Schritt des Zählens der magnetisch reagierenden Teilchen umfasst.
 20. Verfahren nach einem der Ansprüche 17 bis 19, **dadurch gekennzeichnet, dass** der Anlegeschritt den Schritt der Orientierung des Gefäßes umfasst, um die ferromagnetische Fangkonstruktion so unter der Kammer anzurorden, dass die magnetisch reagierenden Teilchen unter dem Einfluss der Schwerkraft zu der Fangkonstruktion transportiert werden.
 21. Verfahren nach einem der Ansprüche 17 bis 19, **dadurch gekennzeichnet, dass** der Anlegeschritt das Anlegen eines externen magnetischen Gefälles an das Gefäß zum Transport der magnetisch reagierenden Teilchen zur Fangkonstruktion umfasst.

22. Verfahren zur Immobilisierung mikroskopischer, magnetisch reagierender Teilchen, die in einem flüssigen Medium suspendiert sind, für Beobachtungszwecke, umfassend folgende Schritte: Bereitstellen eines Gefäßes mit einer Kammer mit einer durchsichtigen Wand und einer ferromagnetischen Fangkonstruktion, die auf der Wand festgehalten wird; Einführen des flüssigen Mediums in die Kammer; Anlegen eines Magnetfelds mit einem externen Gefälle an die Kammer, um ein internes magnetisches Gefälle in der Nähe der ferromagnetischen Fangkonstruktion zu induzieren und magnetisch reagierende Teilchen zur Fangkonstruktion zu transportieren; Auffangen der magnetisch reagierenden Teilchen in einer linearen Monoschicht gegen die Wand und neben der ferromagnetischen Fangkonstruktion; und Beobachten der magnetisch reagierenden Teilchen, die neben der ferromagnetischen Fangkonstruktion gegen die Wand gehalten werden.
- 10 23. Verfahren zur Differenzierung biologischer Substanzen zumindest einer ersten und zweiten Art in einer Flüssigkeitsprobe, umfassend folgende Schritte: Markieren der ersten und zweiten Art von biologischen Substanzen mit entsprechenden Markern zur Erzeugung deutlich unterscheidbarer optischer Eigenschaften; Hinzufügen einer Ferroflüssigkeit mit Bindungsaffinität für biologische Substanzen der ersten und zweiten Art zu der Flüssigkeitsprobe; Überführen der Flüssigkeitsprobe in ein Trenngefäß mit einer durchsichtigen Wand, die eine Seite einer Kammer definiert und einer ferromagnetischen Fangkonstruktion, die an der Wand festgehalten wird; Positionieren des Gefäßes in einem Magnetfeld zur Induzierung eines internen Gefäßes in der Nähe der Fangkonstruktion, das ausreicht, um die biologischen Substanzen der ersten und zweiten Art entlang der Wand neben der Fangkonstruktion zu immobilisieren; und Beobachten der deutlich unterscheidbaren optischen Eigenschaften der ersten bzw. zweiten Art von biologischer Substanz.
- 15 24. Verfahren nach Anspruch 23, **dadurch gekennzeichnet, dass** der Markierungsschritt folgendes umfasst: Hinzufügen erster und zweiter optischer Marker zu der Flüssigkeitsprobe zur Erzeugung deutlich unterscheidbarer optischer Reaktionen von biologischen Substanzen einer ersten Art bzw. einer zweiten Art; und dass der Beobachtungsschritt den Schritt des Beleuchtens des Gefäßes mit optischer Strahlung zur Erzeugung der deutlich unterscheidbaren optischen Reaktionen umfasst.
- 20 25. Verfahren nach Anspruch 23 oder 24, **dadurch gekennzeichnet, dass** die ersten und zweiten biologischen Substanzen Zelltypen von Leukozyten-Unterpopulationen sind.
- 30 26. Verfahren nach Anspruch 25, **dadurch gekennzeichnet, dass** die Leukozyten-Unterpopulationen lebende und tote Leukozyten enthalten.
- 35 27. Verfahren nach Anspruch 23 oder 24, **dadurch gekennzeichnet, dass** die ersten und zweiten biologischen Substanzen erste bzw. zweite Erythrozyten-Unterpopulationen sind.
28. Verfahren nach Anspruch 23 oder 24, **dadurch gekennzeichnet, dass** die ersten und zweiten biologischen Substanzen Zelltypen von Thrombozyten-Unterpopulationen sind.
- 40 29. Verfahren nach Anspruch 23, **dadurch gekennzeichnet, dass** der Beobachtungsschritt die Beobachtung deutlich unterscheidbarer Formen der ersten bzw. zweiten Art von biologischen Substanzen umfasst.
- 30 30. Verfahren nach Anspruch 23, **dadurch gekennzeichnet, dass** die biologischen Substanzen Zelltypen sind und der Beobachtungsschritt das Zählen der immobilisierten Zellen der ersten und zweiten Art und das Bestimmen eines Verhältnisses von immobilisierten Substanzen zur Nettooberfläche der immobilisierten Zellen umfasst.
- 45 31. Verfahren nach Anspruch 23, umfassend folgende Schritte: Verdünnen der Flüssigkeitsprobe mit einem Verdünnungsmittel, das einen fluoreszierenden Membranfarbstoff enthält; und **dadurch gekennzeichnet, dass** der Beobachtungsschritt das Messen der Fluoreszenz von den immobilisierten Zellen zur Bestimmung der proportionalen Oberfläche der immobilisierten Zellen umfasst.
- 50 32. Verfahren nach einem der Ansprüche 23 bis 31, umfassend den Schritt der Durchleitung sequenzieller Ströme von Reagenzien durch das Gefäß zur Erzeugung der deutlich unterscheidbaren optischen Reaktionen.
- 55 33. Verfahren nach Anspruch 32, **dadurch gekennzeichnet, dass** die biologischen Substanzen jeweils Zellen bestimmter Zellunterpopulationen sind, wobei das Verfahren den Schritt der Permeabilisierung der Zellmembranen vor der Durchleitung der sequenziellen Ströme von Reagenzien durch das Gefäß umfasst.

34. Verfahren nach Anspruch 32 oder 33, **dadurch gekennzeichnet, dass** die sequenziellen Ströme von Reagenzien sequenzielle Ströme von Reagenzien zur Erzeugung deutlich unterscheidbarer fluoreszierender optischer Reaktionen umfassen und dass der Beobachtungsschritt die Beleuchtung der immobilisierten Zellen mit einer Beleuchtung, die ausgewählt ist, um die deutlich unterscheidbaren fluoreszierenden Reaktionen zu erzeugen, umfasst.
- 5 35. Verfahren nach Anspruch 23, **dadurch gekennzeichnet, dass** die biologische Substanz Zellen umfasst und der Beobachtungsschritt das Durchführen einer Hybridisierung *in situ* der immobilisierten Zellen umfasst.
- 10 36. Verfahren zur Identifizierung immunophenotypischer Unterpopulationen von biologischen Substanzen, die in einem flüssigen Medium suspendiert sind, umfassend folgende Schritte: Hinzufügen einer Menge magnetisch reagierender Teilchen mit Bindungsaffinität für die biologischen Substanzen zu dem flüssigen Medium; Hinzufügen mehrerer Sonden mit voneinander unterscheidbaren Fluorochromen zu dem flüssigen Medium; Überführen des flüssigen Mediums in ein Trenngefäß mit einer durchsichtigen Wand, die eine Seite einer Kammer definiert und mit einer ferromagnetischen Fangkonstruktion, die an der Wand festgehalten wird; Positionieren des Gefäßes in einem Magnetfeld zur Induzierung eines internen Gefäßes in der Nähe der Fangkonstruktion, das ausreicht, um die biologischen Substanzen entlang der Wand neben der Fangkonstruktion zu immobilisieren; Beleuchten des Gefäßes mit optischer Strahlung, die zumindest ein ausgewähltes Fluorochrom anregen kann; und Beobachten der optischen Reaktion des ausgewählten Fluorochroms.
- 15 37. Verfahren zur Differenzierung von Erythrozyten-Unterpopulationen in einer Flüssigkeitsprobe, umfassend die folgenden Schritte: Behandlung von Erythrozyten in der Flüssigkeitsprobe, so dass sie magnetisch reagieren; Überführen des flüssigen Mediums in ein Trenngefäß mit einer durchsichtigen Wand, die eine Seite einer Kammer definiert und mit einer ferromagnetischen Fangkonstruktion, die an der Wand festgehalten wird; Positionieren des Gefäßes in einem Magnetfeld zur Magnetsierung der magnetisch reagierenden Erythrozyten und zur Induzierung eines internen Gefäßes in der Nähe der Fangkonstruktion, das ausreicht, um die Zellen entlang der Wand neben der Fangkonstruktion anzuziehen und zu immobilisieren; und Beobachten der neben der Fangkonstruktion immobilisierten Erythrozyten.
- 20 38. Verfahren nach Anspruch 37, **dadurch gekennzeichnet, dass** der Behandlungsschritt den Schritt der Markierung der Erythrozyten mit magnetisierbaren Teilchen umfasst.
- 25 39. Verfahren nach Anspruch 38, **dadurch gekennzeichnet, dass** die magnetisierbaren Teilchen mit einer charakteristischen Determinante zumindest einer Unterpopulation von Erythrozyten binden können.
- 30 40. Verfahren nach Anspruch 39, **dadurch gekennzeichnet, dass** die magnetisierbaren Teilchen zumindest eine Bindungssubstanz aus der Gruppe CD71, Glycophorin A und Transferrin umfassen.
- 35 41. Verfahren nach einem der Ansprüche 37 bis 40, **dadurch gekennzeichnet, dass** der Beobachtungsschritt das Zählen von Erythrozyten-Unterpopulationen mit entsprechenden voneinander unterscheidbaren Formen umfasst.
- 40 42. Verfahren nach einem der Ansprüche 37 bis 41, umfassend folgende Schritte: Verdünnen der Flüssigkeitsprobe mit einem Verdünnungsmittel, das einen fluoreszierenden Membranfarbstoff enthält; und **dadurch gekennzeichnet, dass** der Beobachtungsschritt das Messen der Fluoreszenz von den immobilisierten Zellen zur Bestimmung der proportionalen Oberfläche der immobilisierten Zellen umfasst.
- 45 43. Verfahren zur Durchführung einer quantitativen Analyse der Zellen in einem ersten flüssigen Medium, umfassend folgende Schritte: Hinzufügen eines ersten Markers mit einer bekannten Menge erster magnetisch reagierender Teilchen zu dem ersten flüssigen Medium; Hinzufügen einer Menge zweiter magnetisch reagierender Teilchen mit Bindungsaffinität für die Zellen zu dem ersten flüssigen Medium; Überführen des ersten flüssigen Mediums in einem magnetischen Separator zum Trennen der magnetisch reagierenden Teilchen von anderen Bestandteilen des ersten flüssigen Mediums; Resuspendieren der getrennten magnetisch reagierenden Teilchen in einem zweiten flüssigen Medium; Zählen der Menge des ersten Markers und der Zellen in dem zweiten flüssigen Medium zum Bestimmen der Konzentration der Zellen in dem ersten flüssigen Medium, wobei der Zählschritt folgende Schritte umfasst: Überführen des zweiten flüssigen Mediums in eine Kammer eines Trenngefäßes mit einer durchsichtigen Wand und einer ferromagnetischen Fangkonstruktion, die an der Wand festgehalten wird; Positionieren dieses Trenngefäßes in einem Magnetfeld zur Erzeugung eines internen magnetischen Gefäßes in der Nähe der ferromagnetischen Fangkonstruktion; und Zählen der gegen die durchsichtige Wand neben der ferromagnetischen Fangkonstruktion immobilisierten Zellen.
- 50 55

44. Verfahren nach Anspruch 43, umfassend folgende Schritte: Hinzufügen eines zweiten Markers mit einer bekannten Menge dritter magnetisch reagierender Teilchen, deren Magnetmoment ungefähr dem der gebundenen Zellen entspricht, zu dem ersten flüssigen Medium; und **dadurch gekennzeichnet, dass** der Zählschritt den Schritt des Zählens der Menge des zweiten Markers in dem zweiten flüssigen Medium als Maß für die magnetische Trennfizienz umfasst.
 45. Verfahren nach Anspruch 43 oder 44, umfassend den Schritt des Vorbereitens des zweiten Markers durch Bereitstellung einer Menge nicht magnetisch reagierender Teilchen mit einem Bindungsmittel für die zweiten magnetisch reagierenden Teilchen.
 46. Verfahren nach Anspruch 43, **dadurch gekennzeichnet, dass** der Zählschritt des Schritt des Bereitstellens des zweiten flüssigen Mediums zu einem Flusszytometer umfasst.
 47. Verfahren zur Messung eines hämatologischen Parameters einer Erythrozyten enthaltenden Flüssigkeitsprobe, umfassend: Verdünnen einer ersten Blut enthaltenden Flüssigkeitsprobe mit einem Verdünnungsmittel mit einer bekannten Menge erster magnetischer Teilchen zum Erhalt einer zweiten Flüssigkeitsprobe; Behandlung der Erythrozyten einer der ersten und zweiten Flüssigkeitsproben, so dass sie magnetisch reagieren; Überführen der zweiten Flüssigkeitsprobe in eine Kammer eines Trenngefässes mit einer durchsichtigen Wand und einer ferromagnetischen Fangkonstruktion, die an der Wand festgehalten wird; Positionieren dieses Trenngefässes in einem Magnetfeld zur Magnetisierung der Erythrozyten und zum Erzeugen eines internen magnetischen Gefälles in der Nähe der ferromagnetischen Fangkonstruktion; und Zählen der gegen die durchsichtige Wand neben der ferromagnetischen Fangkonstruktion immobilisierten Erythrozyten und ersten magnetischen Teilchen.
 48. Verfahren nach Anspruch 47, **dadurch gekennzeichnet, dass** der Behandlungsschritt den Schritt der Verbesserung der Magnetisierbarkeit der Erythrozyten durch Hinzufügen eines zweiten magnetischen Teilchens mit Bindungsaffinität für Erythrozyten zu einer der ersten und zweiten Flüssigkeitsproben umfasst.
 49. Verfahren nach Anspruch 47 oder 48, umfassend den Schritt des Hinzufügens einer bekannten Menge eines dritten magnetischen Teilchens mit einer magnetischen Eigenschaft, die der der durch Behandlung magnetisch reagierenden Erythrozyten entspricht, zu einer der ersten und zweiten Flüssigkeitsproben, und **dadurch gekennzeichnet, dass** der Zählschritt das Zählen der gegen die Fangkonstruktion immobilisierten dritten magnetischen Teilchen umfasst.
 50. Verfahren nach Anspruch 47 bis 49, umfassend den Schritt des Bestimmens auf Basis der Zählung zumindest eines der Parameter Hämoglobingehalt, Hämatokrit, mittleres Zellvolumen, mittlere Hämoglobinkonzentration und Erythrozytenzahl.

Revendications

1. Appareil pour observer des entités microscopiques magnétosensibles en suspension dans un milieu fluide, comprenant :
 - un récipient ayant une paroi transparente et une chambre formée à l'intérieur pour contenir le milieu fluide; une structure de capture ferromagnétique formée sur la paroi transparente au moyen d'un processus lithographique; et
 - un moyen magnétique pour induire un gradient magnétique interne à proximité de la structure de capture ferromagnétique, qui permette d'immobiliser les entités magnétosensibles le long de la paroi adjacente à la structure de capture.
 2. Appareil selon la revendication 1, dans lequel le récipient comprend une entrée de fluide et une sortie de fluide pour guider un flux de fluide à travers le récipient.
 3. Appareil selon la revendication 2, comprenant un moyen d'alimentation en fluide relié à l'entrée de fluide pour délivrer en séquence une pluralité de réactifs à l'entrée de fluide.
 4. Appareil selon l'une quelconque des revendications 1 à 3, dans lequel la structure de capture ferromagnétique comprend une pluralité de lignes ferromagnétiques ayant un diamètre efficace inférieur à 30 µm.

5. Appareil selon l'une quelconque des revendications 1 à 4, dans lequel le moyen magnétique est configuré pour appliquer un gradient externe au récipient afin de transporter des entités qui se trouvent dans le récipient dans la direction de la structure de capture.
- 10 6. Appareil selon l'une quelconque des revendications 1 à 5, comprenant un système d'observation optique automatisé pour détecter et énumérer les entités immobilisées.
- 15 7. Appareil selon la revendication 6, dans lequel le système d'observation automatisé comprend :
 - un détecteur optique pour détecter lesdites entités immobilisées; et
 - un moyen de poursuite pour balayer le détecteur optique parallèlement aux lignes ferromagnétiques.
- 20 8. Appareil pour observer des entités microscopiques magnétosensibles en suspension dans un milieu fluide, comprenant :
 - un récipient ayant une paroi transparente dans lequel est formée une chambre pour contenir le milieu fluide;
 - une structure de capture ferromagnétique supportée sur la paroi transparente;
 - un moyen magnétique pour induire un gradient magnétique interne à proximité de la structure de capture ferromagnétique, qui permette d'immobiliser les entités magnétosensibles le long de la paroi adjacente à la structure de capture; et
 - un système d'observation optique automatisé pour détecter et énumérer les entités immobilisées.
- 25 9. Appareil selon la revendication 8, dans lequel le système d'observation automatisé comprend :
 - un détecteur optique pour détecter lesdites entités immobilisées; et
 - un moyen de poursuite optique pour balayer le détecteur optique le long de la structure de capture.
- 30 10. Appareil selon la revendication 8, dans lequel le système d'observation automatisé comprend un support vibrant pour supporter et faire vibrer sélectivement le récipient.
- 35 11. Appareil selon la revendication 10, dans lequel le support vibrant comprend un vibreur piézoélectrique.
- 40 12. Appareil pour observer des entités microscopiques magnétosensibles en suspension dans un milieu fluide, comprenant :
 - un récipient ayant une paroi transparente dans lequel est formée une chambre pour contenir le milieu fluide;
 - une structure de capture ferromagnétique supportée sur la paroi transparente;
 - un moyen magnétique pour induire un gradient magnétique interne à proximité de la structure de capture ferromagnétique et pour appliquer un gradient externe perpendiculairement à la structure de capture ferromagnétique, qui permette de transporter et d'immobiliser les entités magnétosensibles le long de la paroi adjacente à la structure de capture.
- 45 13. Appareil selon la revendication 12, dans lequel le moyen magnétique comprend une paire d'aimants ayant des surfaces polaires coniques positionnées pour former un intervalle entre elles afin de recevoir le récipient.
14. Appareil selon les revendications 12 et 13, dans lequel la structure de capture ferromagnétique comprend une pluralité de lignes ferromagnétiques ayant un diamètre efficace inférieur à 30 µm.
- 50 15. Appareil selon la revendication 14, dans lequel les lignes ferromagnétiques sont supportées sur la paroi par adhérence.
16. Appareil selon la revendication 15, dans lequel les lignes ferromagnétiques sont formées sur la paroi au moyen d'un procédé lithographique.
- 55 17. Procédé d'immobilisation d'entités magnétosensibles en suspension dans un milieu fluide pour une observation, comprenant les étapes suivantes :

- la mise en oeuvre d'un récipient ayant une chambre avec une paroi transparente et une structure de capture ferromagnétique supportée sur la paroi;
- l'introduction du milieu fluide dans la chambre;
- l'application d'un champ magnétique à la chambre pour induire un gradient magnétique interne à proximité de la structure de capture ferromagnétique; et
- l'observation des entités magnétosensibles maintenues contre la paroi adjacente à la structure de capture ferromagnétique.
- 5 18. Procédé selon la revendication 17, dans lequel la structure de capture ferromagnétique comprend une pluralité d'éléments ferromagnétiques linéaires et ladite étape d'observation comprend l'étape de balayage le long des éléments ferromagnétiques linéaires avec un système de détection optique automatisé.
- 10 19. Procédé selon la revendication 17, dans lequel ladite étape d'observation comprend l'étape d'énumération des entités magnétosensibles.
- 15 20. Procédé selon l'une quelconque des revendications 17 à 19, dans lequel ladite étape d'application comprend l'étape d'orientation du récipient pour positionner la structure de capture ferromagnétique en dessous de la chambre de sorte que les entités magnétosensibles sont transportées vers la structure de capture sous l'influence de la gravité.
- 20 21. Procédé selon l'une quelconque des revendications 17 à 19, dans lequel l'étape d'application comprend l'application d'un gradient magnétique externe au récipient pour transporter les entités magnétosensibles vers la structure de capture.
- 25 22. Procédé d'immobilisation d'entités microscopiques magnétosensibles, comprenant les étapes suivantes :
- la mise en oeuvre d'un récipient ayant une chambre avec une paroi transparente et une structure de capture ferromagnétique supportée sur la paroi;
- l'introduction du milieu fluide dans la chambre;
- 30 l'application d'un champ magnétique ayant un gradient externe à la chambre pour induire un gradient magnétique interne à proximité de la structure de capture ferromagnétique et pour transporter les entités magnétosensibles vers la structure de capture;
- la collecte desdites entités magnétosensibles dans une monocouche linéaire contre la paroi et adjacente à la structure de capture ferromagnétique; et
- 35 l'observation des entités magnétosensibles maintenues contre la paroi adjacente à la structure de capture ferromagnétique.
23. Procédé de différenciation de substances biologiques d'au moins un premier et un deuxième type dans un échantillon de fluide, comprenant les étapes suivantes :
- 40 le marquage des premier et deuxième types de substances biologiques avec des marqueurs respectifs pour produire des caractéristiques optiques distinctes;
- l'addition à l'échantillon de fluide d'un ferrofluide ayant une affinité de liaison pour les substances biologiques des premier et deuxième types;
- 45 la mise en place de l'échantillon de fluide dans un récipient de séparation ayant une paroi transparente définissant un côté d'une chambre et ayant une structure de capture ferromagnétique supportée le long de la paroi;
- le positionnement du récipient dans un champ magnétique pour induire un gradient interne à proximité de la structure de capture suffisant pour immobiliser les substances biologiques des premier et deuxième types le long de la paroi adjacente à la structure de capture; et
- 50 l'observation des caractéristiques optiques distinctes des premier et deuxième types respectifs de la substance biologique.
24. Procédé selon la revendication 23, dans lequel ladite étape de marquage comprend :
- 55 l'addition à l'échantillon de fluide de premier et deuxième marqueurs optiques permettant de produire des réponses optiques distinctes à partir de substances biologiques d'un premier type et d'un deuxième type respectifs;
- et dans lequel ladite étape d'observation comprend l'étape d'exposition du récipient à un rayonnement optique

qui est à même de produire lesdites réponses optiques distinctes.

25. Procédé selon la revendication 23 ou 24, dans lequel les première et deuxième substances biologiques sont des types de cellules de sous-populations de leucocytes respectives.

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26. Procédé selon la revendication 25, dans lequel les sous-populations de leucocytes comprennent des leucocytes vivants et morts respectifs.

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27. Procédé selon la revendication 23 ou 24, dans lequel les première et deuxième substances biologiques sont des première et deuxième sous-populations respectives de globules rouges.

28. Procédé selon la revendication 23 ou 24, dans lequel les première et deuxième substances biologiques sont des types de cellules de sous-populations respectives de plaquettes.

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29. Procédé selon la revendication 23, dans lequel ladite étape d'observation comprend l'observation de formes distinctes des premier et deuxième types respectifs des substances biologiques.

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30. Procédé selon la revendication 23, dans lequel lesdites substances biologiques sont des types de cellules et ladite étape d'observation comprend la numération des cellules immobilisées des premier et deuxième types et la détermination d'un rapport de substances immobilisées par rapport à la surface nette des cellules immobilisées.

31. Procédé selon la revendication 23, comprenant les étapes suivantes :

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la dilution de l'échantillon de fluide avec un diluant contenant un colorant formant membrane fluorescente; et

26. dans lequel ladite étape d'observation comprend la mesure de la fluorescence issue des cellules immobilisées pour déterminer la surface proportionnée des cellules immobilisées.

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32. Procédé selon l'une quelconque des revendications 23 à 31, comprenant l'étape d'acheminement de flux séquentiels de réactifs à travers le récipient pour produire les réponses optiques distinctes.

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33. Procédé selon la revendication 32, dans lequel les substances biologiques sont des cellules respectives de sous-populations de cellules distinctes, le procédé comprenant l'étape de perméabilisation des membranes des cellules avant d'acheminer les flux séquentiels de réactifs à travers le récipient.

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34. Procédé selon la revendication 32 ou 33, dans lequel les flux séquentiels de réactifs comprennent des flux séquentiels de réactifs pour produire des réponses optiques fluorescentes distinctes et dans lequel ladite étape d'observation comprend l'illumination des cellules immobilisées par une illumination choisie pour produire lesdites réponses fluorescentes distinctes.

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35. Procédé selon la revendication 23, dans lequel ladite substance biologique comprend des cellules et ladite étape d'observation comprend la réalisation in situ d'une hybridation des cellules immobilisées.

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36. Procédé d'identification de sous-populations immunophénotypiques de substances biologiques en suspension dans un milieu fluide, comprenant les étapes suivantes :

l'addition au milieu fluide d'une quantité de particules magnétosensibles ayant une affinité de liaison pour les substances biologiques;

l'addition au milieu fluide d'une pluralité de sondes ayant des fluorochromes distincts respectifs;

la mise en place du milieu fluide dans un récipient de séparation ayant une paroi transparente définissant un côté d'une chambre et ayant une structure de capture ferromagnétique le long de la paroi;

le positionnement du récipient dans un champ magnétique pour induire un gradient interne à proximité de la structure de capture suffisant pour immobiliser les substances biologiques le long de la paroi adjacente à la structure de capture;

l'exposition du récipient à un rayonnement optique pour être à même d'exciter au moins un des fluorochromes choisis; et

l'observation de la réponse optique du fluorochrome choisi.

37. Procédé pour différencier des sous-populations de globules rouges dans un échantillon de fluide, comprenant les étapes suivantes :

- 5 on rend les globules rouges de l'échantillon de fluide magnétosensibles;
on place l'échantillon de fluide dans un récipient de séparation ayant une paroi transparente définissant un côté d'une chambre et ayant une structure de capture ferromagnétique supportée le long de la paroi;
on positionne le récipient dans un champ magnétique pour magnétiser les globules rouges magnétosensibles et pour induire un gradient interne à proximité de la structure de capture suffisant pour attirer et immobiliser les cellules le long de la paroi adjacente à la structure de capture; et
10 on observe les globules rouges immobilisés adjacents à la structure de capture.

38. Procédé selon la revendication 37, dans lequel l'étape visant les globules rouges magnétosensibles comprend l'étape de marquage des globules rouges par des particules magnétisables.

15 39. Procédé selon la revendication 38, dans lequel les particules magnétisables sont à même de se lier à un déterminant caractéristique d'au moins une sous-population de globules rouges.

40. Procédé selon la revendication 39, dans lequel les particules magnétisables comprennent au moins une substance de liaison choisie dans le groupe constitué de la CD71, de la glycophorine A et de la transferrine.

20 41. Procédé selon l'une quelconque des revendications 37 à 40, dans lequel l'étape d'observation comprend la numérotation des sous-populations de globules rouges ayant des formes distinctes respectives.

42. Procédé selon l'une quelconque des revendications 37 à 41, comprenant les étapes suivantes :

25 la dilution de l'échantillon de fluide avec un diluant contenant un colorant formant membrane fluorescente; et dans lequel ladite étape d'observation comprend la mesure de la fluorescence des cellules immobilisées pour déterminer la surface proportionnée des cellules immobilisées.

30 43. Procédé de réalisation d'une analyse quantitative de cellules dans un premier milieu fluide, comprenant les étapes suivantes :

35 l'addition au premier milieu fluide d'un premier marqueur comprenant une quantité connue de premières particules magnétosensibles;
l'addition au premier milieu fluide d'une quantité de deuxièmes particules magnétosensibles ayant une affinité de liaison pour les cellules;
la mise en place du premier milieu fluide dans un séparateur magnétique pour séparer les entités magnétosensibles d'autres composants du premier milieu fluide;
40 la remise en suspension des entités magnétosensibles séparées dans un deuxième milieu fluide;
la numérotation de la quantité du premier marqueur et des cellules dans le deuxième milieu fluide pour déterminer la concentration des cellules dans le premier milieu fluide, ladite étape de numérotation comprenant les étapes suivantes :

45 la mise en place du deuxième milieu fluide dans une chambre d'un récipient de séparation ayant une paroi transparente et une structure de capture ferromagnétique supportée le long de la paroi;
le positionnement du récipient de séparation dans un champ magnétique pour générer un gradient magnétique interne à proximité de la structure de capture ferromagnétique; et
la numérotation des cellules immobilisées contre la paroi transparente adjacente à la structure de capture ferromagnétique.

50 44. Procédé selon la revendication 43, comprenant les étapes suivantes :

55 l'addition au premier milieu fluide d'un deuxième marqueur comprenant une quantité connue de troisièmes particules magnétosensibles ayant un moment magnétique à peu près égal à celui des cellules liées; et dans lequel ladite étape de numérotation comprend l'étape de numérotation de la quantité du deuxième marqueur dans le deuxième milieu fluide comme mesure de l'efficacité de la séparation magnétique.

45. Procédé selon la revendication 43 ou 44, comprenant l'étape de préparation du deuxième marqueur en mettant

en oeuvre une quantité de particules non magnétosensibles portant un agent de liaison pour les deuxièmes particules magnétosensibles.

5 **46.** Procédé selon la revendication 43, dans lequel l'étape de numération comprend l'étape de délivrance du deuxième milieu fluide à un cytomètre en flux.

47. Procédé de mesure d'un paramètre hématologique d'un échantillon de fluide contenant des globules rouges, comprenant les étapes suivantes :

10 on dilue un premier échantillon de fluide comprenant du sang avec un diluant contenant une quantité connue de premières particules magnétiques pour obtenir un deuxième échantillon de fluide;
on rend les globules rouges des premier et deuxième échantillons de fluide magnétosensibles;
on place le deuxième échantillon de fluide dans une chambre d'un récipient de séparation ayant une paroi transparente et une structure de capture ferromagnétique supportée le long de la paroi;
15 on positionne le récipient de séparation dans un champ magnétique pour magnétiser les globules rouges et générer un gradient magnétique interne à proximité de la structure de capture ferromagnétique; et
on effectue la numération des globules rouges et des premières particules magnétiques immobilisées contre la paroi transversale adjacente à la structure de capture ferromagnétique.

20 **48.** Procédé selon la revendication 47, dans lequel l'étape visant à rendre les globules rouges magnétosensibles comprend l'étape de renforcement de la capacité magnétique des globules rouges en ajoutant à l'un des premier et deuxième échantillons de fluide une deuxième particule magnétique ayant une affinité de liaison pour les globules rouges.

25 **49.** Procédé selon la revendication 47 ou 48, comprenant l'étape d'addition à l'un des premier et deuxième échantillons de fluide d'une quantité connue d'une troisième particule magnétique ayant une caractéristique magnétique comparable à celle des globules rouges rendus magnétosensibles; et dans lequel ladite étape de numération comprend la numération de troisièmes particules magnétiques immobilisées adjacentes à la structure de capture.

30 **50.** Procédé selon l'une quelconque des revendications 47 à 49, comprenant l'étape de détermination, en se basant sur ladite numération, d'au moins l'un des paramètres de la teneur en hémoglobines, de l'hématocrite, du volume corpusculaire moyen, de la concentration moyenne en hémoglobine et du nombre de globules rouges.

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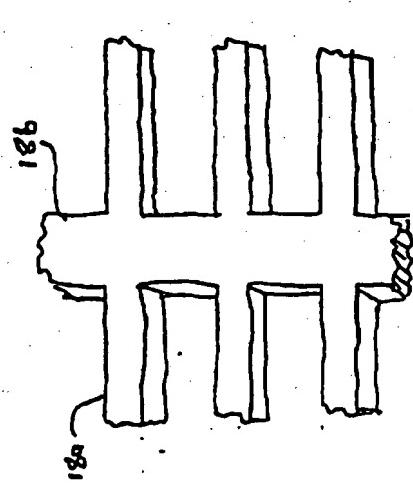


FIG. 2

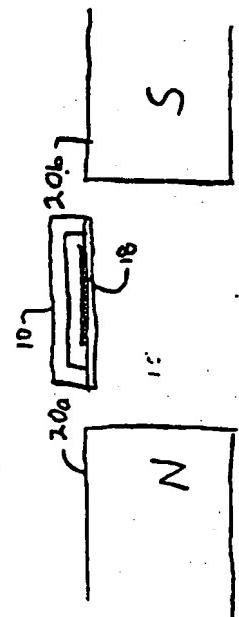


FIG. 3

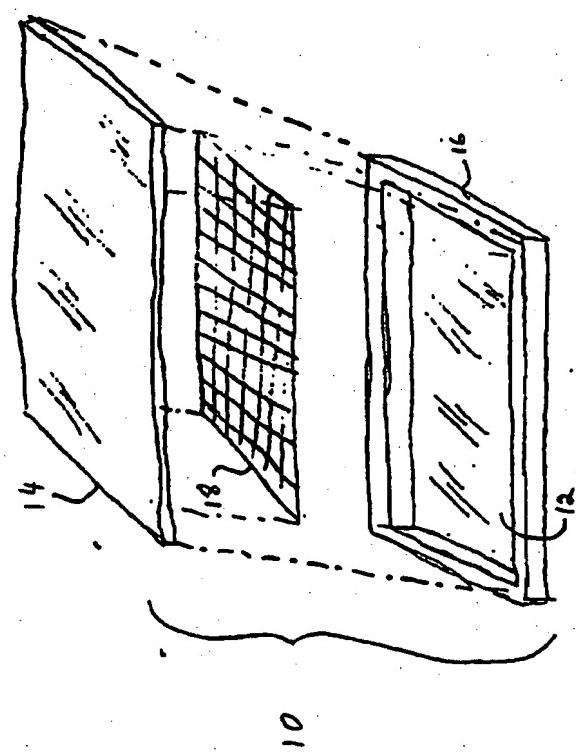


FIG. 1

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FIG 4A

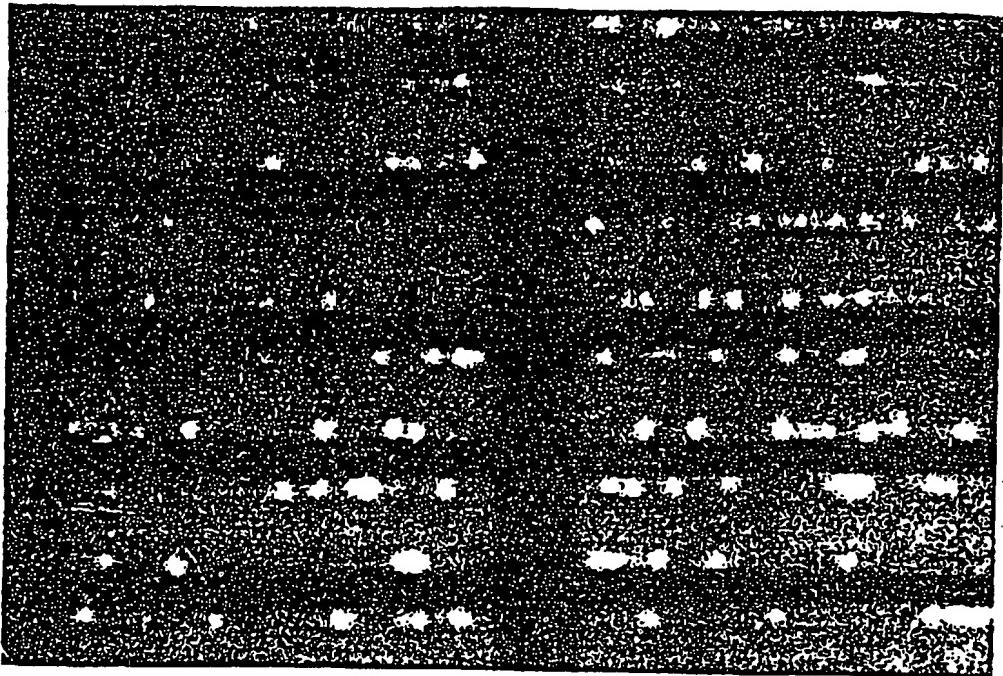
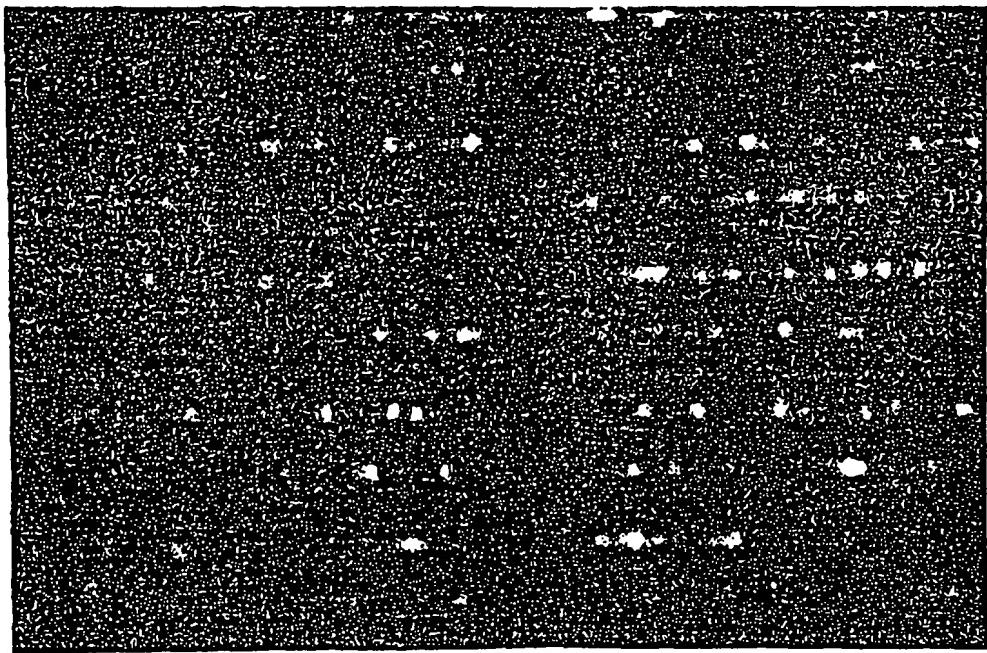


FIG 4B



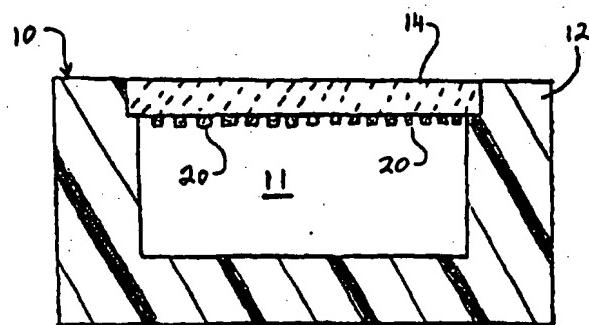
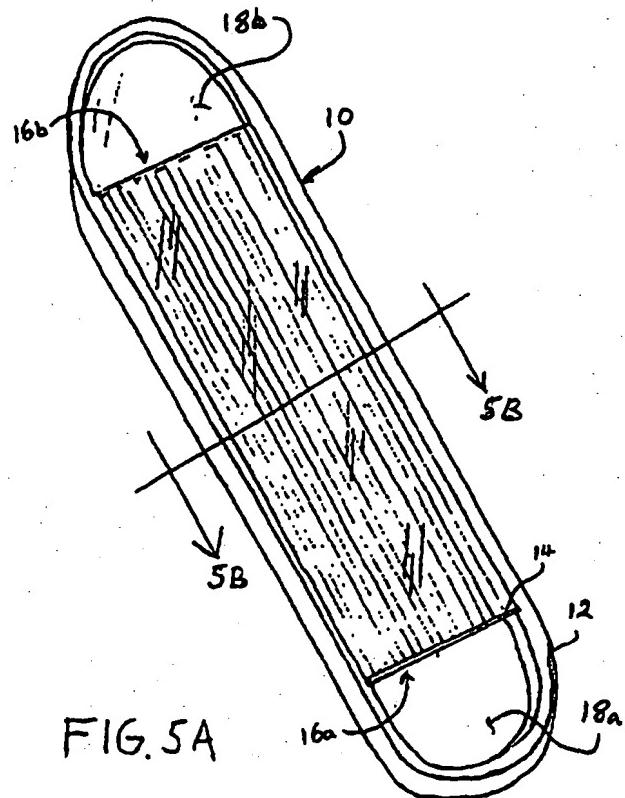
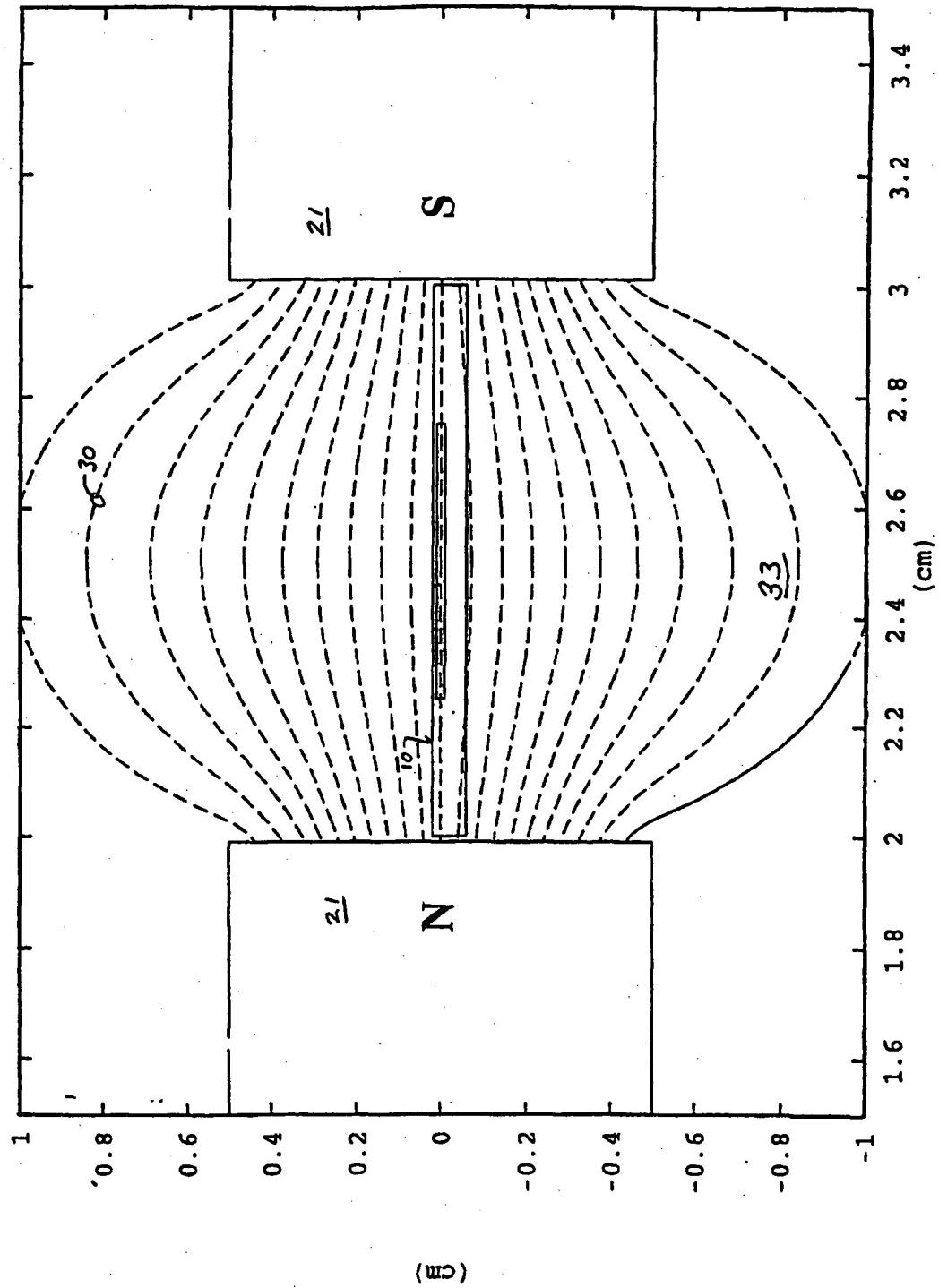
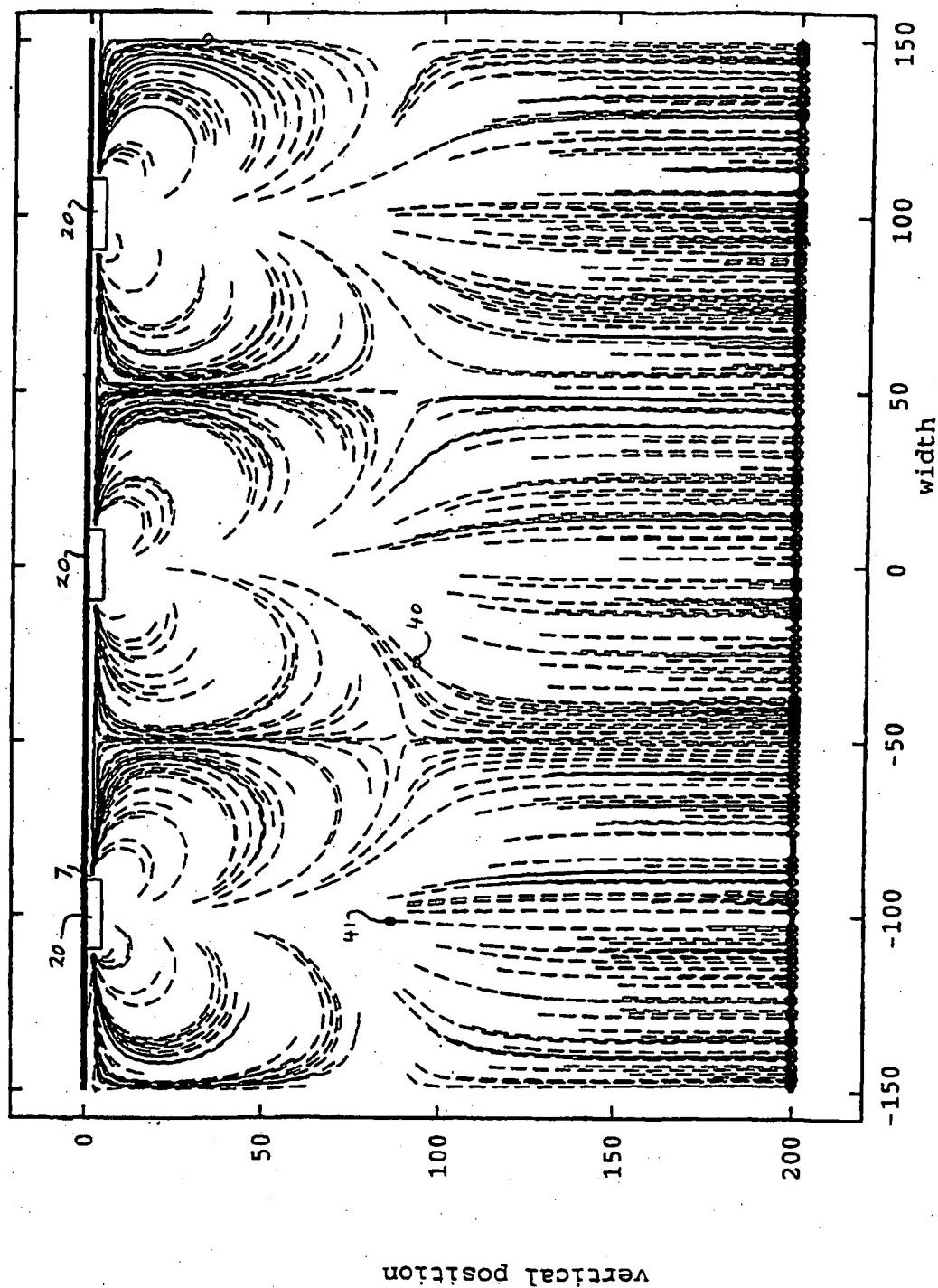


Fig. 6



(E)

Fig. 7



vertical position

Fig. 8

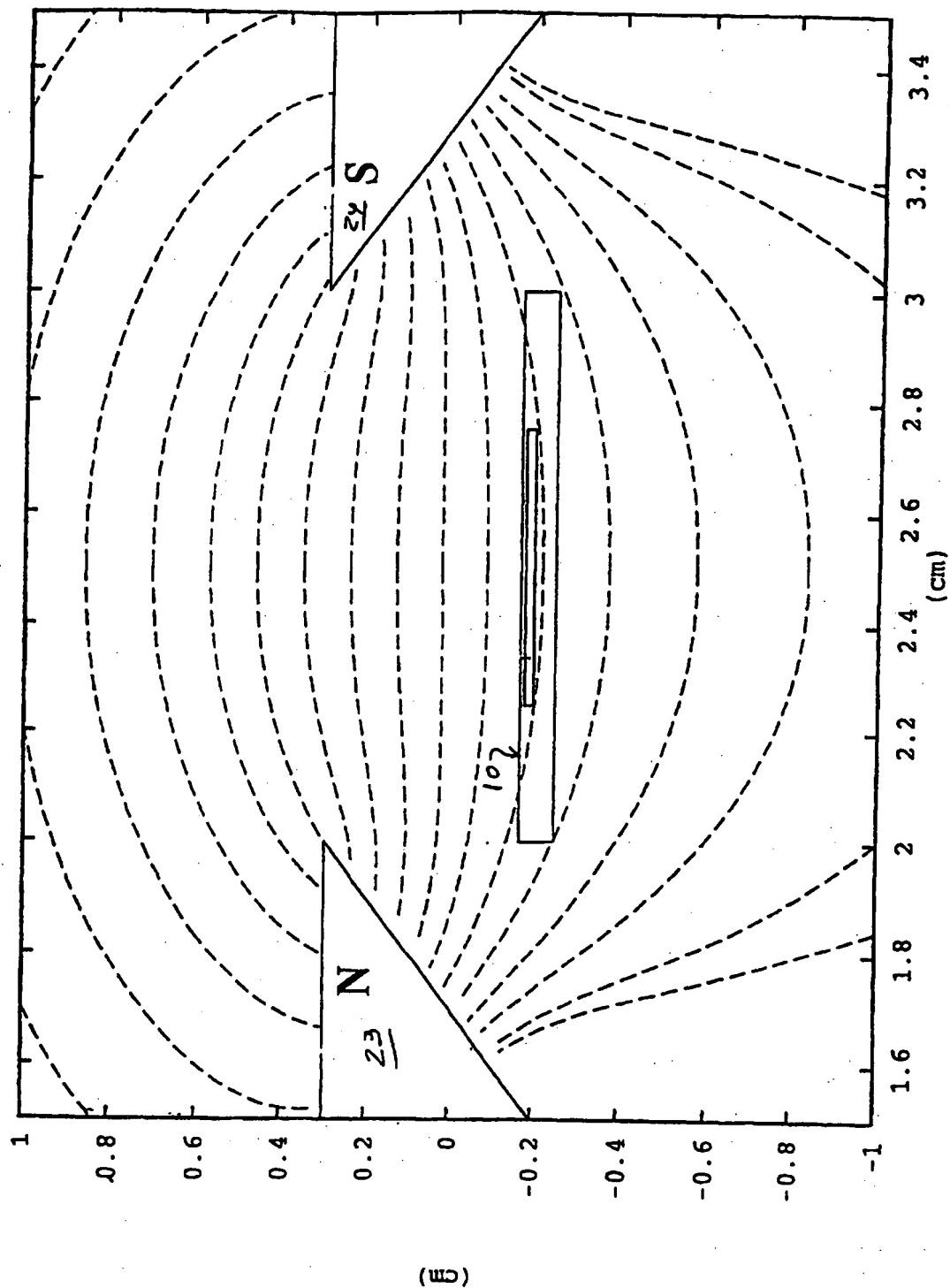


Fig. 9

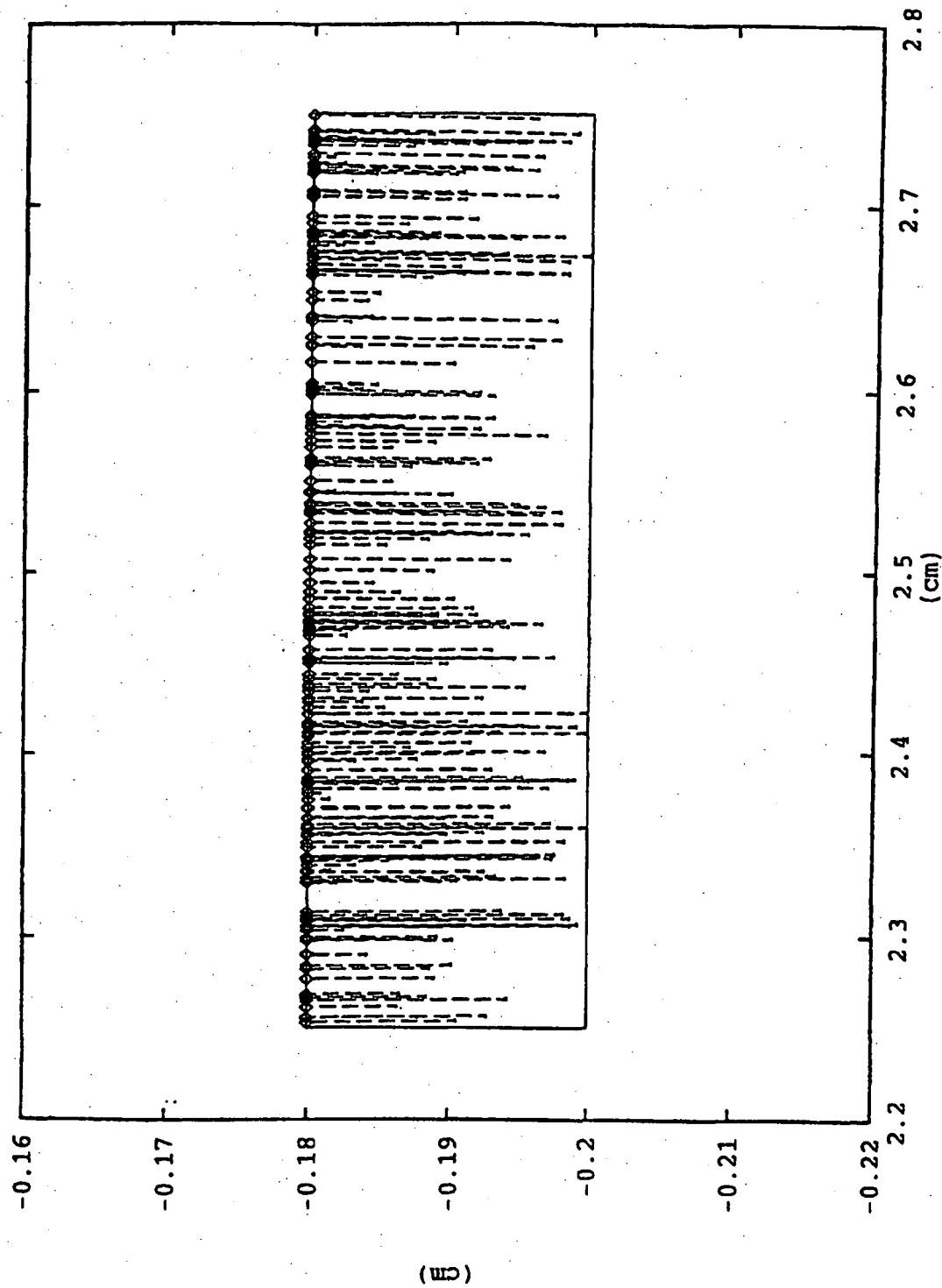
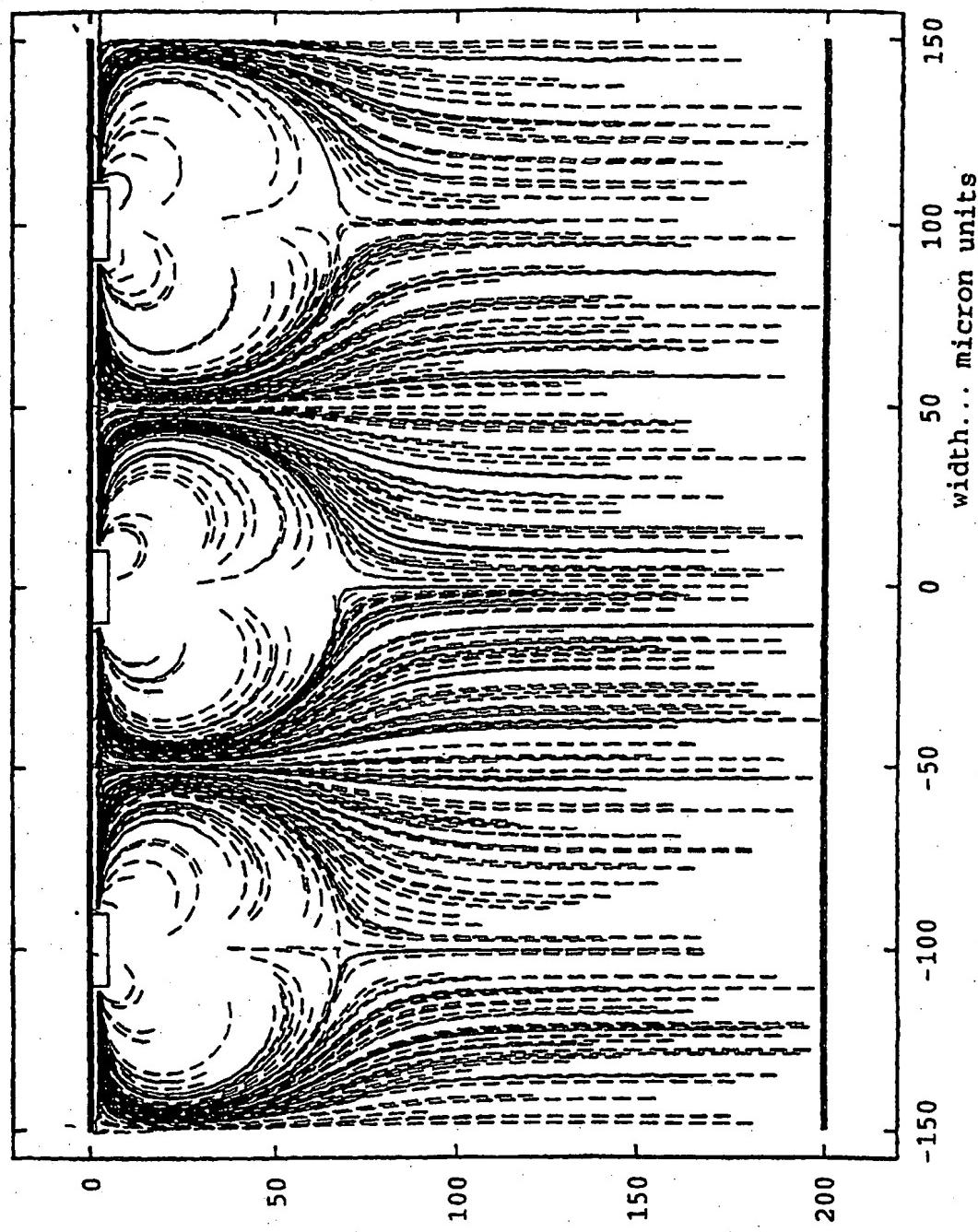


Fig. 10



Vertical Position (microns)

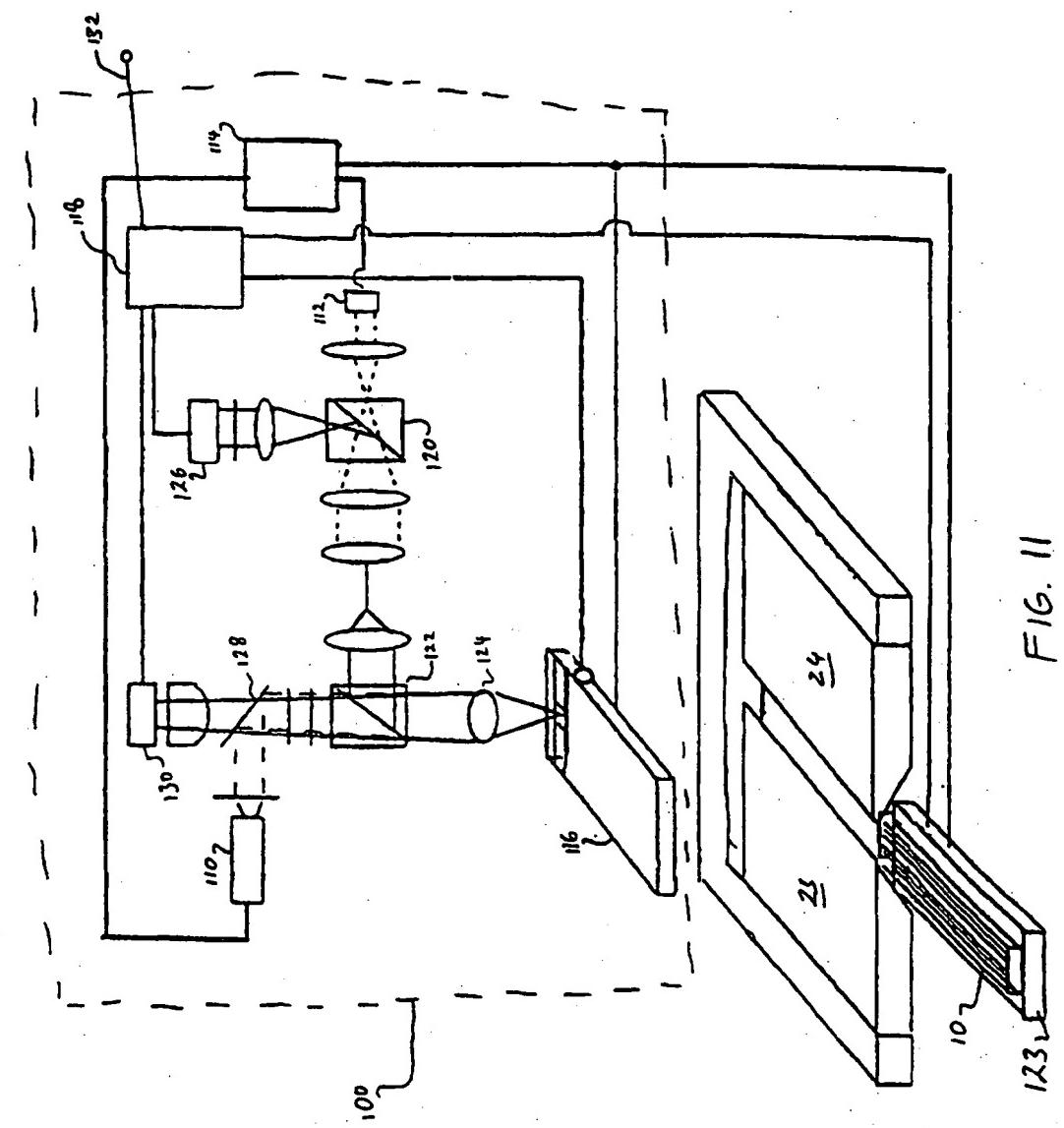


FIG. 11

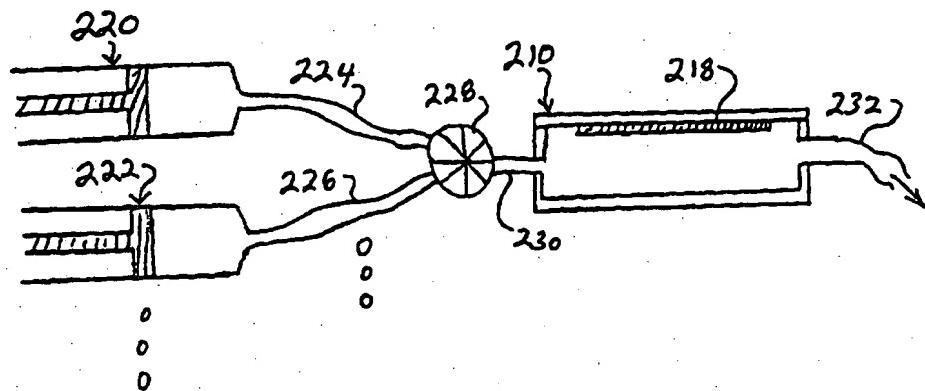
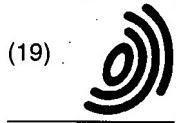


FIG. 12



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(54) APPARATUS AND METHODS FOR CAPTURE AND ANALYSIS OF PARTICULATE ENTITIES

GERÄT UND METHODEN ZUM EINFNAG UND ZUR ANALYSE VON PARTIKEL-EINHEITEN

APPAREIL PERMETTANT LA CAPTURE ET L'ANALYSE D'ENTITES PARTICULAIRES ET
TECHNIQUES CORRESPONDANTES

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| US-A- 5 691 208 | US-A- 5 736 413 |
| US-A- 5 741 714 | |

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Description**FIELD OF THE INVENTION**

[0001] This invention relates to apparatus and methods for the enumeration, examination, and manipulation of particulate entities, especially biological particles such as cells, which involve labelling with magnetically responsive particles.

BACKGROUND OF THE INVENTION

[0002] A magnetic material or magnetic dipole will move in a magnetic field gradient in the direction of increasing magnetic field strength. Magnetic gradients employed in fluid separations are broadly divided into two categories. Internal magnetic gradients are formed by inducing magnetization in a susceptible material placed in the interior of a separation vessel. External gradients are formed by an externally positioned magnetic circuit.

[0003] In the case of a simple rectangular bar magnet, for example, field lines which form magnetic circuits conventionally move from North to South and are easily visualized with iron filings. From this familiar experiment in elementary physics it will be recalled that there is greater intensity of field lines nearest the poles. At the poles, the edges formed at the intersections of the sides and faces of the bar will display an even greater density or gradient. Thus, a steel ball placed near a bar magnet is first attracted to the nearest pole and next moves to the region of highest field strength, typically the closest edge. For magnetic circuits, any configuration which promotes increased or decreased density of field lines will generate a gradient. In opposing magnet designs, such as N-S-N-S quadrupole arrangements, opposing North poles or opposing South poles will have field lines such that in the center of such an arrangement there will be zero field. From the circuits that result from a North pole being opposite to each adjacent South pole, such arrangements generate radial magnetic gradients.

[0004] Internal high gradient magnetic separators have been employed for nearly 50 years for removing weakly magnetic materials from slurries such as in the kaolin industry, or for removing nanosized magnetic materials from solution. (See Kolm, Scientific American, Nov., 1975). In an internal high gradient magnetic separator, a separation vessel is positioned in a uniform magnetic field. A ferromagnetic structure is positioned within the vessel in order to distort the magnetic field and to generate an "internal" gradient in the field. Typically, magnetic grade stainless steel wool is packed in a column which is then placed in a uniform magnetic field which induces gradients on the steel wool as in U.S. Patent No. 3,676,337 to Kolm. Gradients as high as 200 kGauss/cm are easily achieved. The magnitude of the field gradient in the vicinity of a wire is inversely related to the wire diameter. The spatial extent of the high

gradient region is proportionally related to the diameter of the wire. As will be explained below, collection of magnetic material takes place along the sides of the wire, perpendicular to the applied magnet field lines, but not

5 on the sides tangent to the applied field. In using such a system, material to be separated is passed through the resulting magnetic "filter". Then, the collected material is washed, and the vessel is moved to a position outside the field where magnetic materials are removed, allowing the collector to be reused.

[0005] Various attempts have been made to perform continuous (non-cycle) high gradient magnetic separation. Improvements include flow-through devices with fluctuating fields to separate magnetic material from 15 non-magnetic material, as in U.S. Patent No. 3,902,994 to Maxwell. Removable screens of ferromagnetic material are also well known in the art as in U.S. Patent No. 4,209,394 to Kelland. Other flow-through devices are described in U.S. Patent Nos. 4,261,815 and 4,663,029 to Kelland, U.S. Patent No. 4,526,681 to Friedlaender, et al., and commonly owned U.S. patent No. 5,622,831 to Wang & Liberti.

[0006] A method and apparatus for separating cells and other fragile particles is described by Graham, et al 25 in U.S. Patent No. 4,664,796. The apparatus contains a rectangular chamber within a cylinder. One pair of opposing sides of the chamber are made of non-magnetic material, while the other sides are made of magnetic material. The flow chamber is packed with a magnetically responsive interstitial separation matrix of steel wool. The material to be separated is passed through the chamber, which is positioned in a uniform magnetic field. During separation, the chamber is aligned in the magnetic field such that the magnetic sides of the chamber 30 are parallel to the applied field lines, thus inducing a high gradient about the interstitial matrix in the chamber. When the chamber is in this position, magnetically labeled cells are attracted to the matrix and held thereon, while the non-magnetic components are eluted. The chamber is then rotated, so that the magnetic sides face magnets, which "shunts" or "short-circuits" the magnetic field, reclines the gradients in the flow chamber, and allows the particles of interest to be removed by the shearing force of the fluid flow.

[0007] Other internal magnetic separation devices are known. Commonly owned U.S. Patent No. 5,200,084 discloses the use of thin ferromagnetic wires to collect magnetically labeled cells from solution. U.S. Patent No. 5,411,863 to Miltenyi discloses the use of 50 coated steel wool, or other magnetically susceptible material to separate cells. U.S. patent No. 5,662,831 by Liberti and Wang discloses an internal HGMS device useful for the immobilization, observation, and performance of sequential reactions involving cells.

[0008] Turning to the magnetic particles used in such collection devices, over the last twenty years, superparamagnetic materials have become the backbone of magnetic separations technology in a variety of health-

care and bioprocessing applications. Such materials, ranging in size from 25 nm to 100 μm , are characterized in that they are only magnetic when placed in a magnetic field. Once the field is removed, they cease to be magnetic and can normally easily be dispersed into suspension. The basis for superparamagnetic behavior is that such materials contain magnetic cores smaller than 20-25 nm in diameter, which is estimated to be less than the size of a magnetic domain. A magnetic domain is the smallest volume in which a permanent magnetic dipole exists. Magnetically responsive particles can be formed about one or more such cores. The magnetic material of choice is magnetite, although other transition element oxides and mixtures thereof having appropriate particle size exhibit such superparamagnetic behavior.

[0009] Magnetic particles of the type described above have been used for various applications, particularly in health care, e.g. immunoassay, cell separation and molecular biology. Particles ranging from 2 μm to 5 μm are commercially available from Dynal. These particles are composed of spherical polymeric materials into which magnetic crystallites have been deposited. These particles because of their magnetite content and size, are readily separated in relatively low external gradients (0.5 to 2 kGauss/cm). Another similar class of materials are particles manufactured by Rhone Poulenc which typically are produced in the 0.75 μm range. Because of their size, they separate more slowly than the Dynal beads in equivalent gradients. Another class of particulate magnetic material is available from Advanced Magnetics. These particles are basically clusters of magnetite crystals, about 1 μm in size, which are coated with amino polymer silane to which bioreceptors can be coupled. These highly magnetic materials are easily separated in gradients as low as 0.5 kGauss/cm. Due to their size, both the Advanced Magnetics and Rhone Poulenc materials remain suspended in solution for hours at a time.

[0010] There is a class of magnetic particles which has been applied to bioseparations and which have characteristics which place them in a distinct category from those described above. These are nanosized colloids (see U.S. Patent Nos. 4,452,773 to Molday; 4,795,698 to Owen, et al.; 4,965,007 to Yudelson; 5,512,332 to Liberti & Piccoli and U.S. 5,597,531 to Liberti et al. and U.S. patent 5,698,271 to liberti et al.). They are typically composed of single crystal to multi-crystal agglomerates of magnetite coated with polymeric material which make them compatible with aqueous liquids. Individual crystals range in size from 8 to 15 nm. The coatings of these materials have sufficient interaction with solvent water to keep them permanently in a colloidal suspension. Typically, well coated particles below 150 nm will show no evidence of settling for as long as 6 months. These materials have substantially all the properties of ferrofluids.

[0011] Because of the particle size and strong interaction with solvent water, substantial magnetic gradi-

ents are required to separate ferrofluids. It had been customary in the literature to use steel wool column arrangements, such as described above, which generate 100-200 kGauss/cm gradients. However, it was subsequently observed that such materials form "chains" (like beads on a string) in magnetic fields, thus allowing separation in gradient fields as low as 5 or 10 kGauss/cm. This observation led to development of separation devices using large gauge wires which generate relatively low gradients. Large gauge wires can be used to cause ferrofluids to produce uniform layers upon collection. By controlling amounts of ferrofluid in a system, a monolayer can be formed. Magnetically labeled cells can thus be made to form monolayers as described in commonly owned U.S. Patent Nos. 5,186,827 and 5,466,574.

[0012] Analysis of the cellular composition of biological fluids is used in the diagnosis of a variety of diseases. Microscopic examination of cells smeared or deposited on slides and cytochemically stained has been the traditional method for cell analysis. Introduction of impedance based cell counters in the late 1950's has led to a major advance in the accuracy of cell enumeration and cell differentiation. Since then various other technologies have been introduced for cell enumeration and differentiation such as Flowcytometry, Fluorescence Activated Cell Sorting, Quantitative Buffy Coat Analysis, Volumetric Capillary Cytometry, Laser Scanning Cytometry and various image analysis systems. Fluorescence based flowcytometry has dramatically improved the ability to discern different cell types in heterogeneous cell mixtures. This technique is commonly used, for example, to measure the absolute and relative number of cells in a specific subset of leukocytes in blood. In practice, a blood sample is drawn and incubated with a fluorescently labeled antibody specific for this subset. The sample is then diluted with a lysing buffer, optionally including a fixative solution, and the dilute sample is analyzed by flow cytometry. This procedure for analysis can be applied to many different cell surface antigens.

Simultaneous assessment of multiple parameters of individual cells which pass the measurement orifice of a flow cytometer at a speed of up to 1,000 to 10,000 cells/sec is indeed a powerful technology. However, there are limitations on this technology, such as the inability to conveniently accommodate high cell concentrations (e.g., blood needs to be diluted), impracticality of the detection of infrequent or rare cells, and the inability to re-examine the cells of interest. In such situations, the time needed for the flow cytometer to analyze these samples becomes extremely long, thus decreasing the sample throughput. In addition, the settling of cells in the sample tube will occur during this time and require continuous mixing of the sample. To overcome these limitations, clinical samples to be analyzed are typically subjected to various enrichment techniques such as erythrocyte lysis, density separation, immunospecific selection or depletion of cell populations prior to analysis by flow cytometry.

[0013] Coated surfaces have been used for many research and clinical applications over the last several decades. Coated plates, reaction vessels, and tubes are well-known in the art. Coated surfaces have found particular use in immunoassays, and have seen wide usage since the 1960's, starting with radioimmunoassays. Coated cup assays remain common in many of the clinical analyzers in laboratory use today, such as the VITROS ECI, Cyber-fluor, Delfia and the ES-300 systems. See also U.S. Patent 4,376,110. Advantages of coated cup assays include that they provide a single, essentially homogeneous layer of analyte for analysis, which will withstand vigorous wash steps and result in low non-specific binding.

[0014] Commonly owned WO 97/07243, based on U.S. patent application 08/516,694 to Rao & Liberti, relates to the use of a coated surface combined with a magnetic immunoassay. Magnetically collected material is immobilized on a coated surface through a specific binding pair reaction. The specific binding pair is borne upon the magnetic particle, which results in the non-re-orientation of the magnetically collected material, when such particles are perturbed by sample removal or buffer addition and removal. The formation of a monolayer of magnetic material upon the coated surface is also facilitated, which reduces trapping of potentially signal-interfering substances. As a result, resuspension during washing or during signal readout is not required. Additionally, since the binding pair is adhered to the magnetic particle, all magnetic particles become bound to the coated surface, which prevents the loss of particles during washing, resulting in higher signal. However, in the separation of other biological material, such as cells, other concerns must be taken into account. Sample sizes are generally larger than in immunoassay, requiring larger volumes of magnetic material. Additionally, cells are much larger than the analytes in immunoassays, thus requiring larger amounts of magnetic particle reagents in order to convey the cell in the magnetic field. Increased amounts of such reagents are also required to drive the binding reaction, resulting in significant excesses of magnetic material. The sample volume reduction needed to concentrate the cells for analysis also results in an increase in the concentration of magnetic particle reagents. Such a large excess of material tends to obscure the microscopic examination of cells and inhibits their further analysis.

[0015] Other cytological techniques involve cell deposition on a slide for microscopic analysis. Sample preparation for these techniques include cell centrifuges, the Cytoshuttle, sorting of cells by Fluorescence Activated Cell Sorting (FACS), or other cytometric techniques by which target cells are separated after analysis/identification, such as B-D's FACS Sort. Cell centrifuges are sold by several companies, including Shandon Lipshaw and StatSpin. In these systems, a centrifuge is used to deposit a cell sample on a microscope slide. However, drawbacks of the system include cell loss in the centrif-

agation step and the inability to selectively deposit target cells onto a slide. A pre-selection of cells is required in such cases. A pre-selection is also required for the Cytoshuttle, available from Cancer Diagnostics, which uses a filter to collect cells on special filter paper. The cells collected on the filter paper are then transferred to a microscope slide for analysis. Cell loss is also a problem with the Cytoshuttle.

[0016] Cell sorting additions to flow cytometers have been sold by Becton-Dickinson (B-D), Coulter, and Ortho. Shapiro's Practical Flow Cytometry, (3rd ed. Wiley-Liss, NY, 1995) provides a comprehensive description of the theory behind this apparatus. Basically, there are two types of sorters, droplet and fluidic. The droplet sorters include FACSVantage (B-D), MoFlo (Cytomation) and EPICS (Coulter), which divide the fluid stream into individual charged droplets, some of which contain cells. Charged plates deflect the droplets into one or two streams from which they can be collected. In fluidic sorters, such as the FACS Sort (B-D), a mechanical arm is placed in the fluid stream. When a target cell passes, the arm is extended into the sample stream, capturing the fluid containing the target cell and then moving back to its original position in the fluid stream. Since the arm is placed in the fluid stream, a continuous flow of fluid is collected along with the target cell, resulting in a considerable dilution of the target cells.

[0017] The ability to deposit target cells in specific position for analysis has been described by Stovel & Sweet (J Histochem & Cytochem, 27: 284-288 (1979)) and is commercially available as B-D's Accurate Cell Deposition Unit (ACDU). The ACDU is an option added to a droplet sorter which allows sorting of cells into a microtiter well or onto a microscope slide. The slide is guided via computer control to capture target cells on a pre-defined region of the slide. Thus, one is able to sequester individual cells on a portion of a microscope slide, although it is difficult to maintain cell integrity and morphology. However, in the Stovel & Sweet system, the cells are sorted into individual "splash circles" with a 270 micron diameter, making it relatively difficult to locate a single cell within this large diameter. Additionally, not all cells which fall within the sort gate of the scatterplot are actually deposited on the slide, and it is thus impossible to correlate the individual deposited cells to the cells in the sort gate. This problem has been overcome by adding an additional analysis point to determine if the gated cell was actually successfully sorted. This system has been used to index cells sorted into microtiter wells. See Terstappen et al., "Characterization of Human Primitive Hematopoietic Stem Cells", presented at Joint International Workshop on Foetal and Neonatal Hematopoiesis and Mechanisms of Bone Marrow Failure, April 3-6, 1995, Paris. Despite these features, droplet sorting is inherently more complex than fluidic sorting and such instruments could not practically be used in a clinical setting, even with highly trained technicians.

[0018] Fluidic sorters of the type described in U.S.

Patent 5,030,002 to North are relatively easy to maintain and operate, but they have their own set of drawbacks. The mechanical arm is positioned in the fluid stream and liquid is continuously collected by the arm. A target cell is caught when the arm moves into the sample stream. The sheath fluid rate thus determines the collection volume. For example, when the contents of a 500 µl sample has to be sorted, it will take approximately 500 seconds (sample flow 1 µl/sec.) and will produce a total volume of about 50 ml (sheath fluid rate of 18 ml/min. and catcher arm rate of 6 ml/min.). Irrespective of the number of captured cells, the volume will be approximately 50 ml.

[0019] From the foregoing discussion, it will be appreciated that a need exists for apparatus and methods which are capable of efficiently and effectively separating magnetically labeled particles, such as cells, from a fluid medium, including whole blood or sheath fluid, resulting in the capture of the cells of interest in a predetermined pattern on a non-magnetic capture surface, such as a microscope cover slip.

SUMMARY OF THE INVENTION

[0020] The present invention provides apparatus and methods for the collection and immobilization of particulate entities, especially those of biological origin, such as cells.

In accordance with the invention the particulate entities are labelled with magnetically responsive particles and collected on a collection surface, followed by immobilization on the collection surface as a result of an interaction between the members of a specific binding pair, one member of which is uniformly or non-uniformly affixed to the collection surface, and the other member of which is associated with the particulate entities sought to be immobilized.

A method according to the invention for the immobilization of non-magnetic target particulate entities on a collection surface via binding between the members of a specific binding pair, said target particulate entities being present in a mixture with at least one other non-magnetic substance, said method comprising:

- (a) attaching magnetically responsive particles to said target particulate entities to form magnetically labelled composites;
- (b) providing a magnetic field;
- (c) disposing said collection surface in said magnetic field, at least a portion of said collection surface having affixed thereto one member of said specific binding pair;
- (d) adhering said magnetically labelled composites to said collection surface under the influence of said magnetic field;

5 (e) subjecting said adhered magnetically labelled composites to conditions promoting reaction between the members of said specific binding pair; and

10 (f) removing any excess magnetically labelled composites and non-magnetic substance present on said collection surface to yield said magnetically labelled composites immobilized on said collection surface;

15 is characterised in that the other member of said specific binding pair is associated with said non-magnetic target particulate entities rather than with said magnetic label, and in that a magnetic field gradient having a predetermined pattern is produced on the portion of said collection surface affixing said other specific binding pair member, whereby said magnetically labelled composites adhere to said collection surface under the influence 20 of said magnetic field gradient and, after the removal of any excess magnetically labelled composites and non-magnetic substance present on said collection surface, said magnetically labelled composites are retained immobilized on said collection surface in said predetermined pattern.

25 Preferably, ferromagnetic localisation means is positioned relative to said collection surface such that said magnetic field gradient having a predetermined pattern is set up.

30 Apparatus according to the invention for immobilization of magnetically labelled composites, formed by attaching magnetically responsive particles to non-magnetic target particulate entities, on a collection surface via binding between the members of a specific binding pair, said apparatus comprising:

35 (a) magnetic means for providing a magnetic field; and

40 (b) collection surface disposed in the magnetic field generated by said magnetic means, at least a portion of said collection surface having affixed thereto one member of said specific binding pair;

45 is characterised in that the member of said specific binding pair affixed to said collection surface is selected so as to form a specific binding pair with a member associated with the non-magnetic target particulate entities, rather than with said magnetically responsive particles, and in that the apparatus also includes

50 (c) localization means operably associated with said collection surface in said magnetic field for producing a magnetic field gradient having a predetermined pattern on the portion of said collection surface affixing the member of said specific binding pair, whereby in use of the apparatus said magnetically labelled composites are caused to adhere to

said collection surface under the influence of said magnetic field gradient and to become immobilized on said collection surface in said predetermined pattern when subjected to conditions promoting reaction between the members of said specific binding pair.

Preferably, the localisation means is ferromagnetic localisation means.

[0021] As will appear from the following description, the present invention offers a number of notable advantages over existing analytical techniques for separation and analysis of particulate analytes, e.g., cells or microbes, within test samples such as bodily fluids, culture fluids or samples from the environment, which may contain other non-magnetic components. One distinct advantage of the present invention is maintenance of the target entities intact and/or viable upon separation to permit analysis, identification or characterization of the target entities.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022]

FIGURE 1 shows a representative embodiment of the present invention in which a strip of unused staples disposed on a magnet is used as a ferromagnetic cell alignment means.

FIGURE 2A shows another embodiment of the invention using magnets in a bucking array to provide a ferromagnetic localization means. FIGURE 2B depicts a top, perspective view of an apparatus for the collection of cells labelled with magnetically responsive material utilizing the "bucking array" of magnets depicted in Fig. 2A. FIGURE 2C is a side elevation view of Fig. 2B.

DETAILED DESCRIPTION OF THE INVENTION

[0023] The instant invention provides for the collection and immobilization of particulate entities of a target analyte, such as cells, in a predetermined pattern on a non-magnetic collection surface. The term "target analyte", as used herein, includes a variety of substances of potential biological or medical interest which may be measurable individually or as a group. Representative examples of "target analytes" include cells, both eukaryotic (e.g., leukocytes, erythrocytes or fungi) and prokaryotic (e.g., bacteria, protozoa or mycoplasma), viruses, cell components, macromolecules and the like. Often it is desirable to determine the presence or quantity of a particular cell type for diagnostic or therapeutic purposes. Examples include the determination of leukocytes within a population of blood cells, helper T lymphocytes within a population of lymphocytes, fetal cells within maternal circulation, virus-infected cells within a

population of uninfected and infected cells, or neoplastic cells within a population of normal and neoplastic cells. Although the method of this invention is useful for the determination of many different types of target analyte, it will be exemplified hereinbelow with particular reference to the detection of human blood cells.

[0024] The non-magnetic collection surface, preferably a microscope cover slip, is coated with a specific binding substance, that specifically binds a characteristic determinant of the target cells. The collection surface is placed on a magnet and the target cells, which are conveyed to the collection surface under the influence of the magnetic field become immobilized thereon as a result of the binding interaction with the specific receptor for a characteristic cell surface antigen of the target cell, a coating of which receptor is provided on the collection surface. When the surface is removed from the magnetic field, any excess magnetic material, such as magnetic labels that are not bound to specific binding substances affixed to the collection surface, or any non-magnetic substance may be removed from the surface, e.g., by washing. The invention thus allows for the removal of magnetic material not actually bound to target cells, improving the visualization of the target cells by microscopic means and improving conditions for the further culturing or other use of the cells.

[0025] In a particularly preferred embodiment of the invention, the collection surface is placed on a magnet which, due to pole piece design or some other magnetic gradient phenomenon, provides a "ferromagnetic localizing means" which causes an ordered arrangement (a predetermined pattern) of magnetically labelled cells to form on the collection surface. The target cells may be present in a biological specimen such as whole blood, leukopheresis product, bone marrow, or other bodily fluid, as well as cultured cells. The test sample contains magnetically labeled target cells, as well as excess magnetic particles and, usually, non-target material. When the test sample is placed on the collection surface, the magnetically labeled target cells immediately migrate to the region of the surface in registry with the patterned magnetic field gradient produced by the ferromagnetic localizing means. Thus, the magnetically labelled cells will assume the shape, arrangement, or pattern produced by the ferromagnetic localizing means. The target cells which are magnetically conveyed to the collection surface become immobilized thereon as a result of binding interaction with the specific receptor for a characteristic cell surface antigen of the target cell, a coating of which receptor is provided on the collection surface. When the surface is removed from the magnetic field, any excess magnetic material, such as magnetic labels that are not bound to specific binding substances affixed to the collection surface, or any non-magnetic substance may be removed from the surface, e.g., by washing, leaving a defined pattern of target material immobilized on in a predetermined portion of the collection surface. In embodiments wherein the collection surface

is provided by a microscope cover slip and the particulate analyte of interest constitutes cells, staining techniques such as (Immuno) cytochemistry, *in situ* hybridization, or *in situ* PCR can be applied to search for the presence or absence of components of interest. The ordered array of cells will greatly facilitate their microscopic examination and reduce the time required to scan the microscope slide for the presence of the cells of interest. The morphology of the cells is retained throughout the entire process.

[0026] The term "localization", as used herein, refers to the ordered arrangement of particulate analyte formed on a predetermined portion of a collection surface. The portion of the collection surface on which particle localizing occurs may be a point, a straight or curved line, an ordered array of straight or curved lines, which may be of any desired pattern or shape, subject to the size constraints of the collection surface. The term "ferromagnetic localization means", as used herein, refers to ferromagnetic material which becomes magnetized in the presence of a magnetic field to attract magnetically responsive particles. It may be in the form of wires, spheres, or textured material. Ferromagnetic material includes iron, nickel, cobalt, alloys of the same, alloys of magnetic rare Earth elements, and other extremely paramagnetic materials. It is also possible to create an extremely finely focused magnetic field without the need of pole pieces, as described above. Magnetic design, including opposing magnetic poles held closely together can create high field gradients in tightly defined regions on an adjacent surface. The term "internal gradients", as used herein, refers to magnetic field gradients induced upon susceptible material when placed in a magnetic field. Electromagnets can also be used to produce magnetic fields useful in practicing the present invention.

[0027] The method of the invention has broad utility in the separation of particulate biological entities which include a wide variety of substances of biological origins including cells, both eukaryotic (e.g. leukocytes, erythrocytes, or fungi) and prokaryotic (e.g. bacteria, protozoa or mycoplasma), viruses, cell components, such as organelles, vesicles, endosomes, lysosomal packages or nuclei, as well as molecules (e.g. proteins) and macromolecules (e.g. nucleic acids - RNA and DNA). The biological entities of interest may be present in samples or specimens of varying origins, including, without limitation, biological fluids such as whole blood, serum, plasma, bone marrow, sputum, urine, other bodily fluids, such as cerebrospinal fluid, amniotic fluid or lavage fluids, as well as tissue homogenates, disaggregated tissue, or cell culture medium. They may also be present in material not having a clinical source, such as sludge, slurries, water (e.g. ground water or streams), food products or other sources. The method of the invention also has utility in the separation of various bacteria and parasites from fecal matter, urine, or other sources.

[0028] The term "determinant" is used here in a broad

sense to denote the molecular contact regions on target substances that are recognized by receptors in specific binding pair reactions. When used in reference to any of the above-referenced biological entities, "determinant" means that portion of the biological entity involved in and responsible for selective binding to a specific binding substance, the presence of which is required for selective binding to occur. The expression "characteristic determinant" is used herein in reference to cells, for example, to signify an epitope (or group of epitopes) that serve to identify a particular cell type and distinguish it from other cell types. Cell-associated determinants include, for example, components of the cell membrane, such as membrane-bound proteins or glycoproteins, including cell surface antigens of either host or viral origin, histocompatibility antigens or membrane receptors.

[0029] The expression "specific binding substance" or "specific binding pair" as used herein refers to any substance that selectively recognizes and interacts with the characteristic determinant on a particulate biological entity of interest, to substantial exclusion of determinants present on biological entities that are not of interest. Among the specific binding substances which may be used in affinity binding separations in accordance with this invention are antibodies, anti-haptens, lectins, peptides, peptide-nucleic acid conjugates, nucleic acids, hormones, growth factors and more specifically Protein A, Protein G, concanavalin A and soybean agglutinin. Specific binding substances can also be covalently attached to members of other specific binding pairs, such as biotin, avidin, streptavidin or other common capture agents. It is important to note that the term "specific binding", as used herein, refers to the binding that occurs between specific binding substances and cell subpopulations, on the one hand, and between specific binding substances and cell subsets, on the other hand. For example, CD4 Mab specifically binds a characteristic determinant on the leukocyte subset known as T-cells, whereas CD45 Mab specifically binds to all leukocytes. HLA class I antigens recognize an even broader range of cells beyond the subpopulation of leukocytes. However, all three substances are considered specific binding substances.

[0030] The term "antibody" as used herein includes immunoglobulins, monoclonal or polyclonal antibodies, immunoreactive immunoglobulin fragments, single chain antibodies, and peptides, oligonucleotides or any combination thereof which specifically recognize determinants with specificity similar to traditionally generated antibodies.

[0031] The term "magnetically responsive particles" as used herein refers to magnetic particles of metallic or organo-metallic composition, optionally coated with polymer, preferably coated with a polymer of biological origin such as BSA. The particles may be linked with an antibody or other specific binding substance to allow them to specifically bind particulate biological entities of interest. Also included within the ambit of "magnetically

"responsive particles" are the complexes resulting from the interaction between particulate biological entity and magnetic particle, which may be optionally bound to a fluorescent label or other detectable label. Suitable magnetic material is manufactured by Dynal, Rhone Poulanc, Miltenyi, Cardinal Associates, Bangs Labs, Ferrofluidics, Polysciences, and Immunicon.

[0032] The preferred magnetic particles for use in carrying out this invention are particles that behave as true colloids. Such particles are characterized by their sub-micron particle size, which is generally less than about 200 nanometers (nm) (0.20 microns), and their stability to gravitational separation from solution for extended periods of time. Such small particles facilitate observation of the target entities via optical microscopy since the particles are significantly smaller than the wavelength of visible light. Suitable materials are composed of a crystalline core of superparamagnetic material surrounded by molecules which may be physically absorbed or covalently attached to the magnetic core and which confer stabilizing colloidal properties. Most typically, the crystalline core is an agglomeration of single crystals, each sufficiently small that they do not contain a complete magnetic domain. When agglomerated via intercrystalline attractive forces, the magnetic core sizes can be orders of magnitude larger than a magnetic domain, but these materials nonetheless remain superparamagnetic. As a consequence, North Pole, South Pole alignment and subsequent mutual attraction/repulsion of these colloidal magnetic particles does not appear to occur even in moderately strong magnetic fields, contributing to their solution stability. Accordingly, colloidal magnetic particles are not readily separable from solution as such even with powerful electromagnets, but instead require a magnetic gradient to be generated within the test medium in which the particles are suspended in order to achieve separation of the discrete particles. Magnetic particles having the above-described properties can be prepared as described in U.S. Patent Nos. 4,795,698; 5,512,332; 5,597,531; 5,698,271.

[0033] The specimen material used in practicing the instant invention may be any liquid or solution which contains the particulate analyte of interest, and is preferably whole blood. A test sample is incubated with antibodies or other specific binding substances, either directly or indirectly linked to magnetic particles for the time required to allow optimal binding of the substance to its corresponding target particle. Optionally, the specimen material may also be incubated with an enzymatic label, a dye, or other detectable label. In addition, a member of a specific binding pair may optionally be used to label the target material. After the target particles have been labeled with the magnetically responsive material, an optional magnetic collection serves to concentrate the target particles. After magnetic collection, the excess non-magnetic (non-target) component(s) of the test sample is (are) aspirated, and the remaining material may be removed from the magnetic field and re-

suspended in a substantially smaller volume. After the target particles have been labeled, a signal amplification reagent may optionally be added to increase the signal generated for eventual detection of the target particles.

5 The most common signal amplification reagents are biotinylated antibodies used with streptavidin labeled enzyme, but other such systems are known in the art. As another option, the enzymatic labels, dyes, detection antibodies may be added for later use, or the specific 10 binding pair labels may be added at the time of signal amplification. Finally, the magnetically labeled target particles are placed upon a collection surface for magnetic localization.

[0034] The surface used in the practice of the instant 15 invention may be of glass, plastic, quartz, or any other material which permits observation of the target biological material. The substrate which provides the collection surface must be non-magnetic and relatively thin. The thickness of the substrate is chosen in relation to 20 the strength of the magnets and ferromagnetic localization means used. The collection surface-bearing substrate is preferably 0.1-0.3 mm thick. A particularly preferred collection surface-bearing substrate is a glass or plastic microscope cover slip. In some instances, it may 25 be desirable use a containment means, such as a gasket disposed on said collection surface to limit loss of sample, especially in the case of a liquid sample more than about 50 µl. The containment means is preferably nonreactive and non-adherent to the target material. After 30 magnetic collection and immobilization, the gasket, for example, is easily removable to efficiently wash the surface. In other instances, it may be desirable to use a chamber which includes a surface for magnetic collection. It is obvious that only the part of the chamber for 35 the magnetic localization used be suitably thin and compatible for observation of the localized target particles. It will also be appreciated that the test sample can be flowed over the collection surface.

[0035] The collection surface is coated with one member 40 of a specific binding pair. Among the specific binding substances which may be used in affinity binding separations are antibodies, anti-haptens, lectins, peptides, peptide-nucleic acid conjugates, nucleic acids, Protein A; Protein G; concanavalin A; soybean agglutinin; hormones; growth factors; avidin; streptavidin; or biotin. Preferred for this purpose are antibodies, which include 45 single chain antibodies and peptides, oligonucleotides or any combination thereof which specifically recognize determinants with specificity similar to traditionally generated antibodies. Particularly preferred are antibodies against cell-surface antigens. The surface may also be coated with a member of a binding pair which inserts 50 into or adheres to a cell membrane via specific chemical reaction with the internal or external aspect of the lipid bilayer such as lipophilic carbocyanide compounds, aminostyryl compounds, or anti-phosphatidyl choline. The surface may also be coated with a substance, such 55 as polylysine, which binds to all cells based on charge.

[0036] The collection surface-bearing substrate is placed on a ferromagnetic localization means such that the coated surface is not hindered from receiving the test sample. The ferromagnetic localization means is a ferromagnetic material which becomes magnetized in the presence of a magnetic field to attract magnetically responsive particles, and has a characteristic shape, such as wires, spheres or other material providing an irregular or coarse surface when closely packed together. The ferromagnetic material used must have a relatively small area which comes into direct contact with the collection surface-bearing substrate. Additionally, the area of the ferromagnetic localization means which comes into contact with the collection surface-bearing substrate will be in registry with the area on the collection surface on which the target material will become immobilized. Thus, if thin lines of target particles are desired, the corresponding area of the ferromagnetic localization means must be in the form of thin lines. Although ferromagnetic localization means providing thin lines for target particle immobilization is the preferred embodiment, many other shapes or patterns are envisioned to be within the scope of this invention. Some examples include single points; intersecting lines; curved lines; arrays of points, shapes or lines; simple or intricate patterns; and letters, numbers, logos or other indicia. When the ferromagnetic localization means is placed on a magnet and the collection surface bearing substrate is placed on the ferromagnetic localization means, magnetically labelled target particles collected thereon will be immobilized in the pattern or array corresponding to that of the ferromagnetic localization means. Actual magnets could be used in place of the ferromagnetic localization means, but they are less easily formed into the fine shapes contemplated by the present invention. However, it is possible to create the extremely finely focused magnetic field by bringing magnets with opposing poles close together to create high gradients in precisely defined regions of an adjacent collection surface. One such embodiment includes a so called bucking arrangement where in flat magnets (for example, 1 cm x 2 cm x 0.2 cm thick magnetized through the thickness) are stacked alternately with soft steel of identical dimension. By placing the magnets such that alternating steel segments have magnet poles of the same polarity on each side of the segment, substantial gradients can be achieved which draw the magnetically responsive material to the edges of such arrays. In some cases, the fields created by this arrangement of magnets, rather than magnetic pole pieces, may be preferred, since the magnetic reach is greater, thus allowing the use of thicker collection surface-bearing substrates.

[0037] The test sample is placed on the collection surface for a time sufficient for the magnetically labelled target particle to become adhered thereto and for the specific binding pairs to form bonds. With appropriately strong magnetic fields and sufficiently responsive magnetic particles, adherence of the magnetically labelled

target particles should occur on the order of thirty seconds. The time required for specific bond formation will depend on the type of specific binding pair used. In the case of antibodies, binding time will depend on the affinity of the antibody and the antigen density. Although diffusion of the target material to the collection surface and specific bond formation theoretically could occur before magnetic adherence, experience has shown that magnetic adherence is generally faster, provided the magnetic field is sufficiently strong. Additionally, magnetic adherence speeds immobilization by bringing the target particle bearing one member of the specific binding pair into close physical proximity to the collection surface coated with the other member of the specific binding pair.

[0038] After a time sufficient for magnetic adherence and bond formation, the collection surface-bearing substrate may be removed from the ferromagnetic localization means, and the surface may be washed to remove excess, unbound magnetic material and/or non-target material. The non-target material may be material which was inadvertently trapped in the initial collection, and which due to a lack of specific binding substance is not bound to said surface. The non-target material may also be material which was bound to magnetically responsive particles, but which does not bear the specific binding substance required for immobilization. Optional treatment(s) with additional substances to label or stain the target particles is (are) appropriate at this point. Other sequential reactions involving the target particles may also be performed. It is also possible to subject the target particles to conditions appropriate for the removal of the magnetic label from the target entities. Many such methods are well known to those skilled in the art.

[0039] After washing and optional staining steps, the target material will be immobilized on the collection surface for examination. The orderly arrangement of target particles will greatly facilitate their examination. In the case of microscopic examination, less time will be required to scan the microscope slide for the presence of target material. In the case of cells, enumeration, examination and manipulation are possible.

[0040] Figure 1 illustrates a representative apparatus according to this invention, including one embodiment of ferromagnetic localization means. A magnet 1 is shown with a strip of unused, chisel-pointed staples 2 with the chisel points resting upon the magnet 1. A coated microscope cover slip 3 rests on the staple strip 2 awaiting the deposit of the test sample.

[0041] An alternate embodiment of the invention is illustrated in Figures 2A-C. In this instance, magnets 10 are stacked alternately with bars 11 of soft steel which serve as magnetizable spacers. The magnets 10 are assembled with the opposing polarity indicated in the drawing. The magnets and steel spacers are held together by band 13 and thus provide the magnetic field and the ferromagnetic localization means. This assembly can generate a magnetic field gradient in the range

of 30-60 K Gauss. The collection surface-bearing substrate 12 shown is intended to represent a microscope cover slip. As can be seen in Figure 2B, the coverslip 12 rests directly on the ferromagnetic localization means. A non-magnetic, inert gasket 22 is used to contain the liquid test sample on the area of the collection surface in registry with the ferromagnetic localization means for optimal collection. Magnetically labeled cells will collect along lines 21. Figure 2C shows deposition of a test sample onto the collection surface in the area bounded by gasket 22.

[0042] In one embodiment of the invention, a microscope cover slip can be used to collect and immobilize cells for analysis. Preferably, the cells are aligned in a series of parallel lines for easy microscopic examination. Ferromagnetic localization means, such as specially manufactured coarse iron chips with raised, parallel lines can be used. Another ferromagnetic alignment means is a plurality of standard, single-edge razor blades taped securely together with blades exposed and placed on a strong magnet with the blade edges in contact with the collection surface-bearing substrate. A strip of unused staples as illustrated in Figure 1 is also effective. The cover slip is coated with a cell surface adhesion agent, such as a ligand to a cell-surface receptor such as class I antibody by methods which are well known to those skilled in the art. Thus virtually all cells which come into contact with the coated surface will adhere to it, but any potentially contaminating protein, sera, or nucleic acids or other material which is not of interest can be washed away. In this particular embodiment of the invention, all cells in the specimen material are aligned. The cells are labeled, either directly or indirectly with a magnetic colloid or ferrofluid. Preferably, the labeling is done by direct conjugation of the ferrofluid to a specific binding substance which binds all cells. The specimen material in this embodiment includes cultured cells or cells purified from an original mixture by non-magnetic means. Also included may be a cell mixture which one may desire to analyze in its entirety. An optional concentration step includes centrifugation of the starting cell mixture.

[0043] After labeling with the ferrofluid, the cell mixture is placed on the coated collection surface, which is disposed on the ferromagnetic localization means. Alternatively, the cell mixture may be deposited on the collection surface, which is then placed on the ferromagnetic localization means. The magnetically labeled material will collect in registry with the ferromagnetic localization means and the specific binding substance will bind the cells to the surface. Then the cover slip may be removed from the magnet and washed by successively dipping it in wash solutions, including dyes or enzyme substrates. The cover slip is mounted on a microscope slide and examined with a light microscope. The cells are observed in precisely defined parallel lines. With appropriate selection of the ferromagnetic localization means, the lines may be slightly narrower than the field

of view of the microscope for the magnification desired. This allows the microscope stage to traverse rapidly up and down along the lines to examine the cells, either manually or mechanically.

- 5 **[0044]** In another embodiment of the invention, a microscope cover slip can be used to collect and immobilize subsets of human white blood cells for analysis. The cover slip may be coated with a specific binding substance which binds to a relatively broad subpopulation of cells, for example CD45. The magnetic colloid or ferrofluid may be directly labeled with an antibody directed toward a characteristic surface antigen of a cell subset of interest, such as CD4 or CD8 (T-lymphocyte cells), CD56 or CD16 (NK cells), CD19 (B-lymphocytes), CD14 (monocytes), CD83 (dendritic cells), CD33 or CD47 (granulocytes), CD34 (progenitor cells), CD90w (hematopoietic stem cells), CD71 (immature erythrocytes or fetal nucleated erythrocytes), MAb 330 (trophoblasts), EPCAM (epithelial cells), CD31 (endothelial cells), Vimentin (mesenchymal cells) and S-100 (neural cells). In this embodiment of the invention, the specimen material is whole blood, collected by standard venipuncture. An optional initial magnetic separation may be employed to concentrate the original sample and isolate the target cells. The blood is incubated in a test tube with the ferrofluid labeled for a time sufficient for binding of the ferrofluid to the target cells. The test tube is then placed in an external magnetic field, such as described by U.S. Patent 5,186,827. After a collection period sufficient to collect the magnetically labeled cells, the liquid in the tube is aspirated with a pipet. Then the test tube is removed from the magnetic field and the magnetically labelled cells are resuspended in a small amount of buffer. If the starting volume of blood was 5 ml, 200-300 ul of buffer is sufficient to resuspend the target white blood cells. An optional wash and magnetic recollection to remove excess red blood cells or to aid in the resuspension of the cells is possible before final resuspension into the buffer. After the final resuspension, the entire cell suspension may be placed on the surface of the appropriately coated cover slip which has been placed upon ferromagnetic alignment means as described above. The magnetically labeled material will be localized by the ferromagnetic localization means and the specific binding substance will bind the cells to the collection surface. After removal from the magnetic field and the optional washes, the cover slip is mounted on a microscope slide and examined with a light microscope. The cells are observed in tightly defined parallel lines.
- 10 **[0045]** In a further embodiment of the invention, the two magnetic selections may be used to select a distinct subset of cells. The ferrofluid may be coated with an antibody which recognizes one set of cellular determinants, while the coating on the surface recognizes a different set of determinants. For example, a ferrofluid may be coated with CD34 to recognize all progenitor cells, followed by collection and immobilization on a cover slip coated with CD90w, which specifically binds stem cells.

In this case, the first selection by ferrofluid labeling is relatively broad and the second selection by surface binding is relatively selective, since the CD90w cells constitute a distinct subset of the CD34 cells. However, it is also possible to select a set of cells which have a set of relatively unrelated determinants. For example, the ferrofluid could be coated with CD4 and the surface could be coated with CD45RO. Thus only cells which are CD4+, CD45RO+ (memory CD4+ T-helper lymphocytes) are collected. In this case, it will be appreciated that the CD4 could be coated on the surface and the CD45RO could be conjugated with the ferrofluid. It is also possible to mix antibodies and to manufacture a ferrofluid with a coating of two or more antibodies. It also may be possible to coat the surface with a mixture of specific binding substances. Any number of combinations of antibodies are possible, depending on the ease of manufacturing a directly coated ferrofluid, ability to coat the substance onto a collection surface, and the usefulness of the selected subset.

[0046] In yet another embodiment of the invention would employ an initial analysis and/or separation by flow cytometer. The magnetically labeled target material could be introduced into a flow cytometer after optional magnetic separation of target material as described previously. After flow cytometric analysis of each cell, the magnetically labeled cell could be deposited upon a coated surface which has been placed upon ferromagnetic localization means. The flow cytometric analysis could optionally include fluidic or droplet sorting before deposition upon the coated surface. Optionally, the coated surface could include a layer or small "puddles" of liquid to protect against evaporation while droplet sorting. Said liquid could include PBS, culture medium, glycerol or the like. The magnetic gradient induced upon the ferromagnetic localization means would act upon the magnetically responsive target cell to collect and immobilize the cell in a prescribed region of the collection surface, thus allowing for quicker acquisition of the cell after the sample has been analyzed by the flow cytometer. Indexing the cell event on the histogram with the actual cell deposited upon the surface would increase the power of this technology.

[0047] In an alternate version of the embodiment of the invention described immediately above, the coated surface could be in constant, processive motion. Thus, a microscope slide having the thickness of a standard cover slip could be used with a ferromagnetic localization means comprising parallel lines or a spiral into or out of the center of the slide. The cells would be aligned in the order of passing through the flow cytometer, thus allowing for indexing of the cell event. Optionally, the coated surface-bearing substrate could be in constant, unidirectional motion, e.g., on a continuous web. Thus, a relatively long coated web could be used to collect and immobilize the target cells therealong, with the collection surface being conveyed past a stationary ferromagnetic localization means, optionally past a washing sta-

tion(s), and ultimately past an observation station. Thus, the cell event can be observed before the entire sample has been analyzed by the flow cytometer, and the individual cells are easily indexed back to the original histogram. Individual cells are also available for further analysis or manipulation.

[0048] Still another embodiment of the instant invention involves aligning a set of cells present in whole blood without an initial magnetic separation step. For example, if one desired to observe the CD4 cells in blood, a sample of whole blood could be incubated with a ferrofluid conjugated to CD4. If a non-magnetic surface were coated with CD3 and placed on a ferromagnetic localization means, then the blood sample may be placed directly on said coated surface. After magnetic collection and immobilization of the target cells and formation of the specific pair bond, the surface could be removed from the magnetic field and washed. Thus, all contaminating cells would be washed away, including platelets and red blood cells.

[0049] The following examples describe the present invention in further detail and set forth the best mode contemplated by the inventors for carrying out the invention, but are not to be construed as limiting the invention.

EXAMPLE 1

EPIHELIAL CELL SELECTION FROM WHOLE BLOOD

[0050] Colloidal magnetic particles were prepared as described in U.S. Patent 5,698,271. The particles were further conjugated with an antibody which specifically binds to a cell surface protein encoded by the GA73.3-2 gene.

[0051] Whole blood was collected from a healthy donor by venipuncture in a 7 ml purple-top Vacutainer® tube. A 5 ml sample of blood was measured with a pipet and placed in an empty 7 ml red-top Vacutainer® tube. 20 µl of cell buffer (isotonic 7 mM sodium phosphate, pH 7.4 with 1% BSA and 50 mM EDTA) containing 125 ± 10 cells from the breast cancer cell line SKBR-3 were added to the blood sample. 2.0 ml of the above-described antibody-conjugated, colloidal magnetic particles was also added to the blood sample and mixed. The final concentration of the magnetic particles was 4.28 µg/ml. The blood sample was allowed to incubate 10-15 min. at room temperature. The Vacutainer tube containing the blood sample was placed in a quadrupole magnetic separation device of the kind described in U.S. Patent 5,186,827. The cavity defined by the four magnets exactly accommodated the dimensions of the tube. The magnetic separation was allowed to proceed for 10 minutes at room temperature. The uncollected material was aspirated and the tube was removed from the quadrupole device. The collected cells were resuspended in 2 ml of cell buffer. The sample container was again

placed in the quadrupole device and allowed to separate for 5 minutes. The uncollected material was aspirated and after removal from the quadrupole device, the target cells were resuspended in 150 µl cell buffer.

[0052] A 1.9 cm x 1.6 cm x 5.0 cm magnet (Crucible Magnetics, Elizabethtown, KY) was used for the immobilization of the magnetically labeled material. The pole pieces used were a strip of unused standard chisel point staples (Stanley Bostich, East Greenwich, RI). A strip of approximately 25 staples were broken off and placed on the magnet, with chisel point touching the magnet. The microscope cover slip was placed so as to overlay the staple strip. The cover slip had previously been coated with an antibody which specifically binds Her^{2/neu}. A rubber gasket with an inner area approximately 1.8 x 1.2 cm was used to contain the sample in registry with the staple strip.

[0053] The entire 150 µl sample of resuspended target cells was placed on the area of cover slip bounded by the rubber gasket. The brown-colored ferrofluid could be seen by visual examination to align with the staples, forming approximately 25 brown lines in the clear liquid drop. The cover slip was washed by dipping in PBS to remove the unbound material. The cover slip was air dried and stained with a cytochemical stain (Wright Giemsa.) The cover slip was then mounted on a microscope slide and examined with a light microscope. Fifty-nine of the SKBR-3 cells originally spiked into the blood were observed immobilized in regions corresponding to the ferromagnetic alignment means.

EXAMPLE 2

SELECTION OF CD4 CELLS BY CD4-FERROFLUID AND IMMOBILIZATION ONTO COVER SLIP USING CD45 MAB

[0054] In this example, a relatively small subset of target cells was selected using the colloidal magnetic particles and immobilized on a cover slip by means of an additional specific binding pair which binds to the subpopulation of cells comprising the above-mentioned subset. A 0.5ml sample of whole blood from the purple-top Vacutainer from Example 1 was added to a 12x75mm polystyrene tube. Added to the blood sample were an aliquot of 0.5ml of colloidal magnetic particles, prepared as described in Example 1, but conjugated to CD4 Mab, and 20 µl of CD45 MAb-biotin (2 µg), and the sample was incubated for 15 minutes at room temperature. The final concentration of the magnetic particles was 7.5 µg/ml. The tube was then transferred to a quadrupole magnetic separation device, as described in Example 1, and the separation was allowed to proceed for 10 minutes at room temperature. The uncollected material was aspirated and the tube was removed from the device. The collected sample was then resuspended in 1 ml of cell buffer and magnetically separated again, as described herein above, for 5 minutes. The uncollected

sample was aspirated and the collected CD4 cells were resuspended in 150 µl of cell buffer. The test sample was deposited on a streptavidin coated cover slip using the same gasket arrangement described in Example 1 and stained. The cells were seen as several lines without any free magnetic particles when observed under a microscope. In this example, all CD4 positive cells were labeled with colloidal magnetic particles and CD45 MAb-biotin and were bound to the cover slip.

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Claims

1. A method for the immobilization of non-magnetic target particulate entities on a collection surface via binding between the members of a specific binding pair, said target particulate entities being present in a mixture with at least one other non-magnetic substance, said method comprising:

(a) attaching magnetically responsive particles to said target particulate entities to form magnetically labelled composites;

(b) providing a magnetic field;

(c) disposing said collection surface in said magnetic field, at least a portion of said collection surface having affixed thereto one member of said specific binding pair;

(d) adhering said magnetically labelled composites to said collection surface under the influence of said magnetic field;

(e) subjecting said adhered magnetically labelled composites to conditions promoting reaction between the members of said specific binding pair; and

(f) removing any excess magnetically labelled composites and non-magnetic substance present on said collection surface to yield said magnetically labelled composites immobilized on said collection surface;

characterised in that the other member of said specific binding pair is associated with said non-magnetic target particulate entities rather than with said magnetic label, and in that a magnetic field gradient having a predetermined pattern is produced on the portion of said collection surface affixing said other specific binding pair member, whereby said magnetically labelled composites adhere to said collection surface under the influence of said magnetic field gradient and, after the removal of any excess magnetically labelled composites and non-magnetic substance present on said col-

- lection surface, said magnetically labelled composites are retained immobilized on said collection surface in said predetermined pattern.
2. A method as claimed in claim 1, wherein ferromagnetic localisation means is positioned relative to said collection surface such that said magnetic field gradient having a predetermined pattern is set up. 5
3. A method as claimed in claim 1 or 2, wherein said target particulate entities are selected from cells, cell components, bacteria and viruses. 10
4. A method as claimed in claim 1 or 2, wherein said target particulate entities are selected from leukocytes, hematopoietic stem cells, T-lymphocytes, NK cells, B-lymphocytes, monocytes, dendritic cells, granulocytes, progenitor cells, erythrocytes, trophoblasts, epithelial cells, endothelial cells, fetal cells, mesenchymal cells and neural cells. 15 20
5. A method as claimed in any of claims 1 to 4, wherein said specific binding pair is selected from antibody/receptor, single chain antibody/receptor, protein/receptor, peptide/receptor, nucleic acid/receptor, hapten/anti-hapten, anti-lectin/lectin, biotin/avidin, biotin/streptavidin, lipid intercalating compound/lipid bilayer, and negatively charged cell membrane/positively charged surface. 25
6. A method as claimed in any of claims 1 to 5, further including analyzing said immobilized particulate entities by microscopy. 30
7. A method as claimed in any of claims 1 to 5, further including analyzing said immobilized particulate entities by fluorescence cytochemistry. 35
8. A method as claimed in any of claims 1 to 5, further including analyzing said immobilized particulate entities by immunocytochemistry. 40
9. Apparatus for immobilization of magnetically labelled composites, formed by attaching magnetically responsive particles to non-magnetic target particulate entities, on a collection surface via binding between the members of a specific binding pair, said apparatus comprising:
- (a) magnetic means for providing a magnetic field; and 50
 - (b) a collection surface disposed in the magnetic field generated by said magnetic means, at least a portion of said collection surface having affixed thereto one member of said specific binding pair;
- characterised in that** the member of said spe- 55
- cific binding pair affixed to said collection surface is selected so as to form a specific binding pair with a member associated with the non-magnetic target particulate entities, rather than with said magnetically responsive particles, and **in that** the apparatus also includes
- (c) localization means operably associated with said collection surface in said magnetic field for producing a magnetic field gradient having a predetermined pattern on the portion of said collection surface affixing the member of said specific binding pair, whereby in use of the apparatus said magnetically labelled composites are caused to adhere to said collection surface under the influence of said magnetic field gradient and to become immobilized on said collection surface in said predetermined pattern when subjected to conditions promoting reaction between the members of said specific binding pair.
10. Apparatus as claimed in claim 9, wherein said localisation means is ferromagnetic localisation means. 25
11. Apparatus as claimed in claim 10, wherein said ferromagnetic localization means comprises a series of ferromagnetic strips positioned in closely-spaced parallel array within said field. 30
12. Apparatus as claimed in claim 10, wherein said magnetic means comprises a series of magnetic poles and said ferromagnetic localisation means comprises a series of elongated magnetizable spacers disposed between said poles. 35

Patentansprüche

1. Verfahren zur Immobilisierung nichtmagnetischer Zielteilchen auf einer Auffangoberfläche mittels Bindung zwischen den Mitgliedern eines spezifischen Bindungspaares, wobei die Zielteilchen in einer Mischung mit mindestens einer anderen nichtmagnetischen Substanz vorhanden sind, wobei das Verfahren umfasst:
 - (a) Anbringen magnetisch ansprechender Partikel an den Zielteilchen, um magnetisch markierte Verbünde zu bilden;
 - (b) Bereitstellen eines Magnetfelds;
 - (c) Anordnen der Auffangoberfläche in dem Magnetfeld, wobei auf mindestens einem Teil der Auffangoberfläche ein Mitglied des spezifischen Bindungspaares festgehalten wird;
 - (d) Haften der magnetisch markierten Verbünde an der Auffangoberfläche unter dem Ein-

- fluss des Magnetfelds;
- (e) Einwirken von Bedingungen, die die Reaktion zwischen den Mitgliedern des spezifischen Bindungspaares fördern, auf die haftenden, magnetisch markierten Verbünde; und
- (f) Entfernen jeglicher überschüssiger magnetisch markierter Verbünde und nichtmagnetischer Substanz, die auf der Auffangoberfläche vorhanden sind, um die auf der Auffangoberfläche immobilisierten magnetisch markierten Verbünde zu ergeben;
- dadurch gekennzeichnet, dass** das andere Mitglied des spezifischen Bindungspaares mit den nichtmagnetischen Zielteilchen anstatt mit der magnetischen Markierung assoziiert ist und dass ein Magnetfeldgradient mit einem festgelegten Muster auf dem Teil der Auffangoberfläche erzeugt wird, der das andere Mitglied des spezifischen Bindungspaares festhält, wodurch die magnetisch markierten Verbünde unter dem Einfluss des Magnetfeldgradienten auf der Auffangoberfläche haften und nach der Entfernung jeglicher überschüssiger magnetisch markierter Verbünde und nichtmagnetischer Substanz, die auf der Auffangoberfläche vorhanden sind, die magnetisch markierten Verbünde auf der Auffangoberfläche in dem festgelegten Muster immobilisiert gehalten werden.
2. Verfahren nach Anspruch 1, bei dem das ferromagnetische Positionierungsmittel relativ zu der Auffangoberfläche so angeordnet wird, dass der Magnetfeldgradient mit einem festgelegten Muster eingerichtet wird.
 3. Verfahren nach Anspruch 1 oder 2, bei dem die Zielteilchen ausgewählt sind aus Zellen, Zellkomponenten, Bakterien und Viren.
 4. Verfahren nach Anspruch 1 oder 2, bei dem die Zielteilchen ausgewählt sind aus Leukozyten, hämatopoietischen Stammzellen, T-Lymphozyten, NK-Zellen, B-Lymphozyten, Monozyten, dendritischen Zellen, Granulozyten, Zeugungszellen, Erythrozyten, Trophoblasten, Epithelialzellen, Endothelialzellen, fetalen Zellen, Mesenchymalzellen und Neuralzellen.
 5. Verfahren nach einem der Ansprüche 1 bis 4, bei dem das spezifische Bindungspaar ausgewählt ist aus Antikörper/Rezeptor, Single-Chain-Antikörper/Rezeptor, Protein/Rezeptor, Peptid/Rezeptor, Nukleinsäure/Rezeptor, Hapten/Antihapten, Antilektin/Lektin, Biotin/Avidin, Biotin/Streptavidin, Lipid interkalierender Verbindung/Lipiddoppelschicht und negativ geladener Zellmembran/positiv geladener Oberfläche.
6. Verfahren nach einem der Ansprüche 1 bis 5, bei dem die immobilisierten Teilchen des Weiteren mikroskopisch analysiert werden.
7. Verfahren nach einem der Ansprüche 1 bis 5, bei dem die immobilisierten Teilchen des Weiteren durch Fluoreszenzytochemie analysiert werden.
8. Verfahren nach einem der Ansprüche 1 bis 5, bei dem die immobilisierten Teilchen des Weiteren durch Immunozytochemie analysiert werden.
9. Vorrichtung zur Immobilisierung magnetisch markierter Verbünde, die durch Anbringen magnetisch ansprechender Partikel auf nichtmagnetischen Zielteilchen gebildet sind, auf einer Auffangoberfläche mittels Bindung zwischen den Mitgliedern eines spezifischen Bindungspaares,
- wobei die Vorrichtung umfasst:
- (a) Magnetmittel zur Bereitstellung eines Magnetfelds; und
 - (b) eine Auffangoberfläche, die in dem durch das Magnetmittel erzeugten Magnetfeld angeordnet ist, wobei an mindestens einem Teil der Auffangoberfläche ein Mitglied des spezifischen Bindungspaares festgehalten wird;
- dadurch gekennzeichnet, dass** das an der Auffangoberfläche festgehaltene Mitglied des spezifischen Bindungspaares so gewählt ist, dass es ein spezifisches Bindungspaar mit einem Mitglied, das mit den nichtmagnetischen Zielteilchen assoziiert ist, statt mit den magnetisch ansprechenden Partikeln bildet und dass die Vorrichtung auch
- (c) Positionierungsmittel enthält, die in Wirkbeziehung mit der Auffangoberfläche in dem Magnetfeld stehen, um einen Magnetfeldgradienten mit einem festgelegten Muster auf dem Teil der Auffangoberfläche zu erzeugen, der das Mitglied des spezifischen Bindungspaares festhält, wodurch beim Gebrauch der Vorrichtung die magnetisch markierten Verbünde dazu gebracht werden, unter Einfluss des Magnetfeldgradienten an der Auffangoberfläche zu haften und auf der Auffangoberfläche in dem festgelegten Muster immobilisiert zu werden, wenn sie Bedingungen ausgesetzt werden, die die Reaktion zwischen den Mitgliedern des spezifischen Bindungspaares fördern.
10. Vorrichtung nach Anspruch 9, bei der das Positionierungsmittel ist ferromagnetisches Positionierungsmittel ist.
11. Vorrichtung nach Anspruch 10, bei der das ferromagnetische Positionierungsmittel eine Reihe ferromagnetischer Streifen umfasst, die in einer eng be-

abstandeten parallelen Gruppierung in dem Feld angeordnet sind.

12. Vorrichtung nach Anspruch 10, bei der das Magnetmittel eine Reihe magnetischer Pole umfasst und das ferromagnetische Positionierungsmittel eine Reihe länglicher, magnetisierbarer Distanzstücke umfasst, die zwischen den Polen angeordnet sind.

Revendications

1. Procédé pour l'immobilisation d'entités particulières non magnétiques cibles sur une surface de collecte par l'intermédiaire du liage entre les éléments d'une paire de liage spécifique, lesdites entités particulières cibles étant présentes dans un mélange avec au moins une autre substance non magnétique, ledit procédé comprenant les étapes consistant à :
 - (a) attacher des particules magnétiquement sensibles auxdites entités particulières cibles pour former des composites étiquetés magnétiquement ;
 - (b) fournir un champ magnétique ;
 - (c) disposer ladite surface de collecte dans ledit champ magnétique, au moins une partie de ladite surface de collecte ayant fixé sur elle un élément de ladite paire de liage spécifique ;
 - (d) faire adhérer lesdits composites étiquetés magnétiquement à ladite surface de collecte sous l'influence dudit champ magnétique ;
 - (e) soumettre lesdits composites ayant adhéré et qui sont étiquetés magnétiquement à des conditions favorisant la réaction entre les éléments de ladite paire de liage spécifique ; et
 - (f) retirer tout composite étiqueté magnétiquement excédentaire, et toute substance non magnétique excédentaire, présents sur ladite surface de collecte pour donner lesdits composites étiquetés magnétiquement immobilisés sur ladite surface de collecte ;

caractérisé en ce que l'autre élément de ladite paire de liage spécifique est associé auxdites entités particulières non magnétiques cibles plutôt qu'à ladite étiquette magnétique, et **en ce qu'un gradient de champ magnétique ayant un profil prédéterminé est produit sur la partie de ladite surface de collecte fixant ledit autre élément de la paire de liage spécifique, ce par quoi** lesdits composites étiquetés magnétiquement adhèrent à ladite surface de collecte sous l'influence dudit gradient de champ magnétique et, après le retrait de tout composite étiqueté magnétiquement excédentaire et de toute surface non magnétique excédentaire ,présents sur ladite surface de collecte, lesdits composites étiquetés magnétiquement sont retenus à l'état immobilisé

sur ladite surface de collecte dans ledit profil pré-déterminé.

2. Procédé selon la revendication 1, dans lequel un moyen de localisation ferromagnétique est positionné par rapport à ladite surface de collecte de telle façon que ledit gradient de champ magnétique ayant un profil prédéterminé soit établi.
- 10 3. Procédé selon la revendication 1 ou 2, dans lequel lesdites entités particulières cibles sont sélectionnées à partir de cellules, composants de cellules, bactéries et virus.
- 15 4. Procédé selon la revendication 1 ou 2, dans lequel lesdites entités particulières cibles sont sélectionnées à partir de leucocytes, de cellules souches hématopoïétiques, de lymphocytes T, de cellules NK, de lymphocytes B, de monocytes, de cellules dendritiques, de granulocytes, de cellules ancêtres, d'érythrocytes, de trophoblastes, de cellules épithéliales, de cellules endothéliales, de cellules foetales, de cellules mésenchymales et de cellules neurales.
- 20 5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel ladite paire de liage spécifique est sélectionnée parmi les : anticorps/récepteur, anticorps à chaîne unique/récepteur, protéine/récepteur, peptide/récepteur, acide nucléique/récepteur, haptène/anti-haptène, anti-lectine/lectine; biotine/avidine, biotine/streptavidine, composé intercalaire lipide/bicouche lipide, et membrane de cellule chargée négativement/surface chargée positivement.
- 25 6. Procédé selon l'une quelconque des revendications 1 à 5, comprenant en outre l'analyse par microscopie desdites entités particulières immobilisées.
- 30 7. Procédé selon l'une quelconque des revendications 1 à 5, comprenant en outre l'analyse par cytochimie à fluorescence desdites entités particulières immobilisées.
- 35 8. Procédé selon l'une quelconque des revendications 1 à 5, comprenant en outre l'analyse par immuno-cytochimie desdits entités particulières immobilisées.
- 40 9. Appareil pour l'immobilisation de composites étiquetés magnétiquement, formés en attachant des particules magnétiquement sensibles à des entités particulières non magnétiques cibles, sur une surface de collecte par le liage entre les éléments d'une paire de liage spécifique, ledit appareil comprenant :
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(a) un moyen magnétique pour fournir un champ magnétique ; et
(b) une surface de collecte disposée dans le champ magnétique généré par ledit moyen magnétique, au moins une partie de ladite surface de collecte ayant fixé sur elle un élément de ladite paire de liage spécifique ;
caractérisé en ce que l'élément de ladite paire de liage spécifique fixée à ladite surface de collecte est sélectionnée de façon à former une paire de liage spécifique avec un élément associé aux entités particulières non magnétiques cibles, plutôt qu'auxdites particules magnétiquement sensibles, et **en ce que** l'appareil comprend en outre
(c) un moyen de localisation associé en fonctionnement avec ladite surface de collecte dans ledit champ magnétique pour produire un gradient de champ magnétique ayant un profil pré-déterminé sur la partie de ladite surface de collecte fixant l'élément de ladite paire de liage spécifique, ce par quoi lors de l'utilisation de l'appareil, lesdits composites étiquetés magnétiquement sont conduits à adhérer à ladite surface de collecte sous l'influence dudit gradient de champ magnétique et à s'immobiliser sur ladite surface de collecte selon ledit profil pré-déterminé quand ils sont soumis à des conditions favorisant la réaction entre les éléments de ladite paire de liage spécifique.

10. Appareil selon la revendication 9, dans lequel ledit moyen de localisation est un moyen de localisation ferromagnétique.

11. Appareil selon la revendication 10, dans lequel ledit moyen de localisation ferromagnétique comprend une série de lamelles ferromagnétiques positionnées dans des rangées étroitement espacées dans ledit champ.

12. Appareil selon la revendication 10, dans lequel ledit moyen magnétique comprend une série de pôles magnétiques et ledit moyen de localisation ferromagnétique comprend une série d'entretoises allongées pouvant être magnétisées, disposées entre lesdits pôles.

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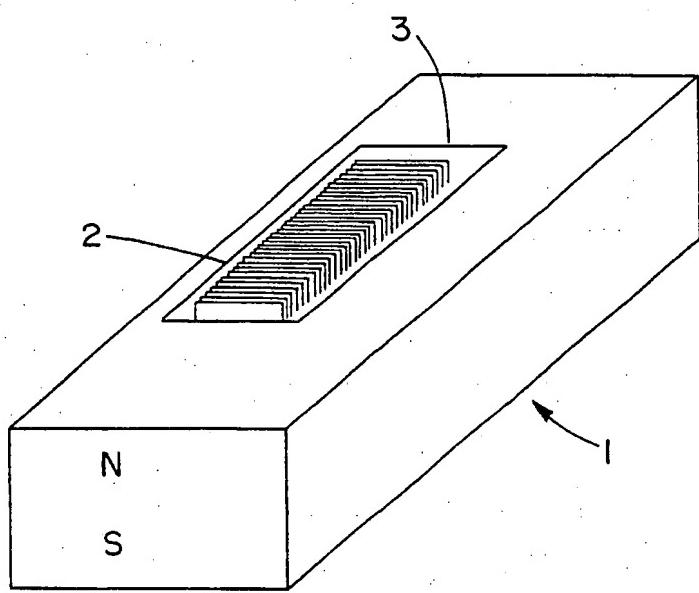


FIG. I

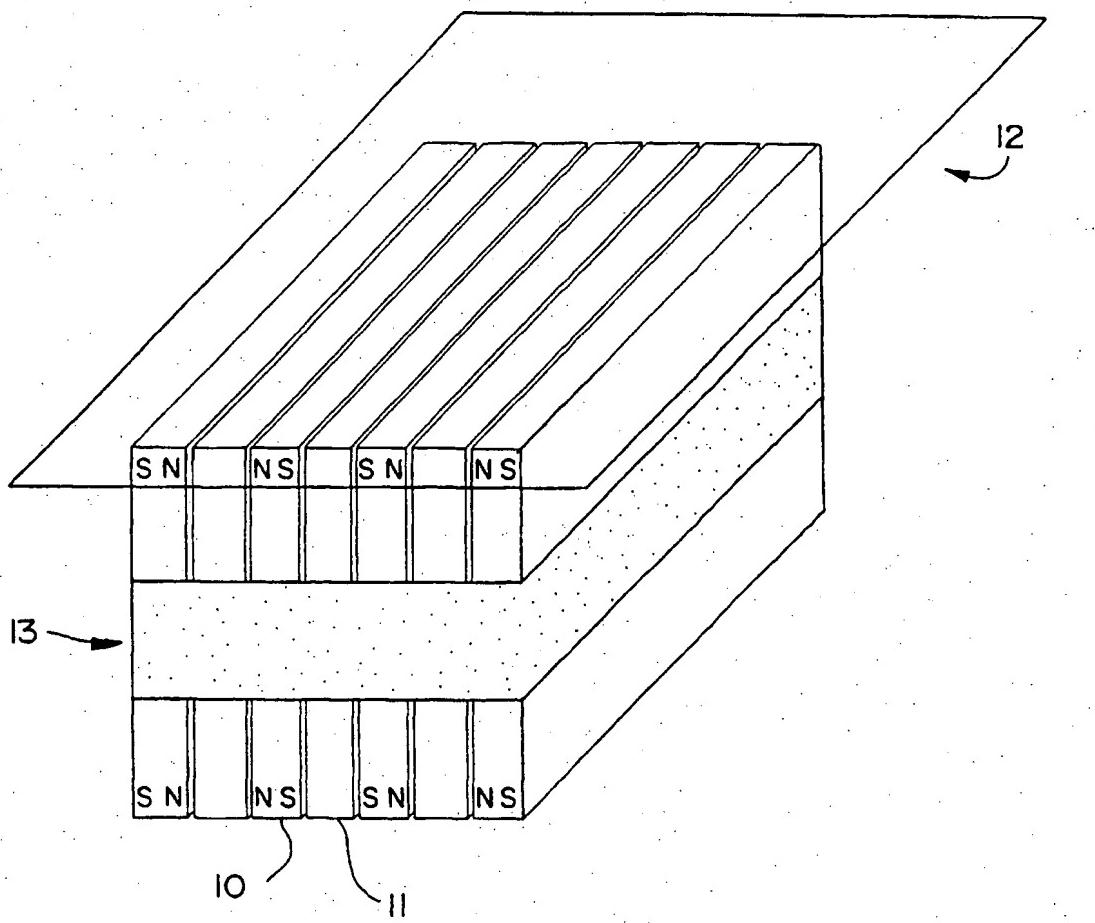


FIG. 2A

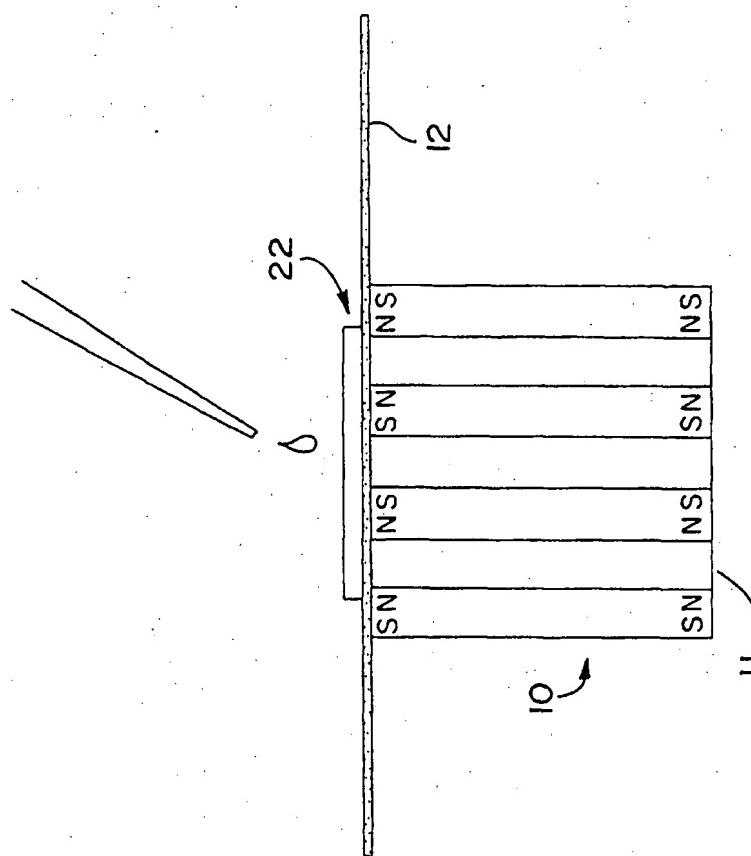


FIG. 2C

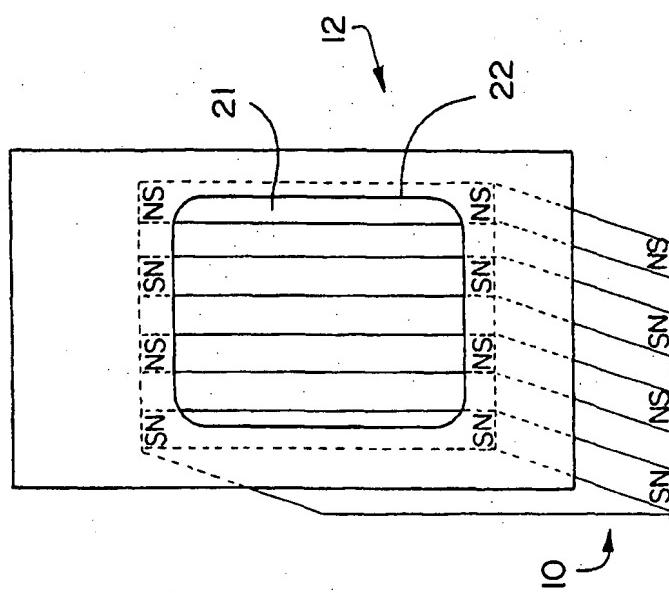


FIG. 2B



(12)

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(54) METHODES ET COMPOSITIONS POUR LA DETECTION D'EVENEMENTS PATHOLOGIQUES

VERFAHREN UND ZUSAMMENSETZUNG ZUR DETEKTION PATHOGENER ZUSTÄNDE

METHODS AND COMPOSITIONS FOR DETECTING PATHOLOGICAL EVENTS

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Remarques:

Le dossier contient des informations techniques présentées postérieurement au dépôt de la demande et ne figurant pas dans le présent fascicule.

Description

[0001] La présente invention concerne de nouvelles compositions et méthodes pour la détection d'événements pathologiques. Elle concerne plus particulièrement des compositions et méthodes de détection d'événements pathologiques à distance. L'invention concerne également des outils, kits et compositions pour la mise en oeuvre de telles méthodes, ainsi que leurs utilisations dans le domaine de la santé humaine ou animale, ou en recherche expérimentale par exemple.

[0002] Avec le vieillissement de la population dans les pays industrialisés, apparaissent de nouveaux besoins en matière de diagnostic. Les maladies telles que les cancers, les maladies neurodégénératives seraient mieux prises en charge pour le bénéfice des patients et de la société si l'on disposait de diagnostics prédictifs de l'apparition et de l'évolution de la pathologie.

[0003] L'expérience montre que plus le diagnostic est posé précocement plus les chances de contrôler l'évolution probable de la maladie sont grandes. C'est le cas, très clairement des cancers. Les campagnes de détection précoce des cancers du sein par mammographie systématique ont permis d'améliorer l'espérance de survie à ces cancers. De même, on peut supposer qu'une prise en compte précoce des patients développant une maladie d'Alzheimer permettrait de ralentir significativement son évolution.

[0004] Ces maladies, telles les cancers et maladies neurodégénératives, ont une incidence qui augmente fortement avec l'âge de la population. Il est probable que ces maladies prennent des années avant de s'installer et de pouvoir être détectées. Il ressort par exemple que l'accumulation d'altérations successives au niveau du génome humain est requise pour aboutir à l'initiation de cancers. De même des études génétiques réalisées dans des populations choisies présentant une incidence forte de maladie d'Alzheimer, associées à des expériences de génétique chez l'animal, soulignent également le caractère multifactoriel de l'initiation de cette pathologie.

[0005] Ces pathologies liées au vieillissement présentent des caractéristiques communes, telles que :

- des altérations cellulaires survenant à la suite de déséquilibres de l'environnement des tissus incriminés liés à des agressions physiques, chimiques ou biologiques ;
- la contribution des cellules du système immunitaire.

[0006] Si les altérations des tissus incriminés ne sont préférentiellement identifiées qu'à la suite de biopsies, il se pourrait que des altérations des cellules immunitaires, reflet d'un développement pathologique en cours, puissent être détectées à distance des foyers de développement de ces pathologies, puisque la plupart des cellules du système immunitaire évoluent entre les tissus et le compartiment sanguin ou les ganglions lymphatiques.

phatiques.

[0007] Les cellules lymphocytaires et les macrophages sont les principaux médiateurs de la réponse immunitaire cellulaire. Lymphocytes et macrophages sont présents dans les tissus ainsi que dans le sang. Ce sont ces cellules qui entrent les premiers en contact avec les tissus étrangers à l'organisme. Ces cellules macrophagiques dégradent les tissus et substances incriminés. Les peptides issus des protéines dégradées sont ensuite fixés par des molécules du système majeur d'histocompatibilité de classe II qui les amènent à la surface du macrophage où ces complexes sont reconnus par les lymphocytes T. D'autres systèmes de présentation de peptides et d'activation des réponses immunitaires existent et ont été décrits notamment dans le cas du développement de cancers.

[0008] Aujourd'hui les diagnostics de ce type de maladies sont faits alors que la pathologie est installée. Pour ce qui est des cancers, par exemple, le diagnostic est posé à partir d'une imagerie médicale et d'un diagnostic morphologique des tissus obtenus à la suite de biopsies. Pour une pathologie comme la maladie d'Alzheimer c'est un faisceau d'observations médicales qui permet de poser le diagnostic.

[0009] Il existe donc un réel besoin de disposer d'outils et de méthodes permettant de dépister de manière précoce, simple et fiable l'apparition de pathologies, notamment de pathologies liées à un dérèglement des mécanismes de régulation des voies de signalisation cellulaire, en particulier des pathologies caractérisées par une hyper-prolifération cellulaire (cancer, dégénérescence nerveuse, sténose, etc).

[0010] L'introduction des techniques issues de la biologie moléculaire, associée à la bio-informatique, a permis de concevoir des librairies (ou banques) de fragments d'ADN caractérisant un état pathologique donné, permettant de déterminer à partir d'un échantillon très petit d'un tissu quelconque, la présence ou l'absence de références pathologiques.

[0011] La présente invention décrit à présent une nouvelle approche pour la détection de pathologies *in vitro*. Plus particulièrement, la présente invention décrit de nouvelles méthodes et compositions pour la détection d'événements pathologiques, notamment de signatures génétiques pathologiques. L'invention décrit en outre des méthodes et compositions utilisables pour la détection à distance d'événements pathologiques, c'est-à-dire sur des matériaux biologiques distincts des tissus pathologiques. Les compositions et méthodes de l'invention offrent à présent aux cliniciens, biologistes et industriels de nouvelles solutions pour le diagnostic *in vitro*, fondées sur des méthodes directes, rapides, sensibles et économiques, et automatisables.

[0012] Plus particulièrement, la présente invention repose notamment sur la mise en évidence qu'il est possible de déterminer, à partir d'échantillons biologiques comprenant des cellules circulantes, la présence ou le risque de développement d'une pathologie. Plus parti-

culièrement, l'invention repose sur la mise en évidence qu'il est possible de détecter dans un échantillon biologique comprenant des cellules sanguines, l'existence d'une pathologie, y compris à des stades très précoce d'initiation ou de développement, auxquels tout autre diagnostic existant serait inefficace.

[0013] Un premier objet de l'invention réside plus particulièrement dans un procédé de détection *in vitro* de la présence d'une pathologie chez un sujet, selon la revendication 1.

[0014] Plus préférentiellement, le procédé de l'invention comprend la détermination de la présence, dans l'échantillon, de cellules sanguines présentant des altérations de l'expression génétique caractéristiques de la présence de la pathologie.

[0015] Ainsi, la présente invention repose d'une part sur l'utilisation de cellules sanguines pour la réalisation d'un test à distance de la présence d'un événement pathologique et, d'autre part, sur l'utilisation de technologies génomiques permettant de détecter des altérations de l'expression (en particulier de la transcription) du génome dans ces cellules.

[0016] Selon une variante particulière, la présente invention comprend donc plus préférentiellement la détermination de la présence, dans un échantillon biologique, de cellules sanguines présentant des altérations transcriptionnelles et/ou post-transcriptionnelles de l'expression génétique, caractéristiques de la présence d'une pathologie.

[0017] L'invention réside en la démonstration qu'il est possible de détecter une pathologie en cours de formation à partir de signatures génomiques identifiées dans les cellules sanguines, les foyers embryonnaires pathologiques pouvant exister dans des tissus nerveux comme le cerveau ou la moelle épinière (sites de maladies neurodégénératives) ou dans tout autre tissu par exemple à l'origine d'un cancer (sein, poumon, prostate, foie, tissu osseux etc.).

[0018] L'invention démontre d'une manière inattendue qu'il existe au niveau des cellules sanguines (préférentiellement des cellules nucléées, telles que lymphocytes, macrophages, monocytes, cellules dendritiques, etc.) des altérations transcriptionnelles et post-transcriptionnelles de l'expression génétique à la suite d'interaction(s) directe(s) ou indirecte(s) avec les cellules en cours d'initiation pathologique.

[0019] Plus particulièrement, l'invention démontre d'une manière inattendue qu'il existe au niveau des cellules sanguines (préférentiellement des cellules nucléées, lymphocytes, macrophages, monocytes, cellules dendritiques, etc.) des altérations de la transcription qualitatives des gènes à la suite d'interaction(s) directe(s) ou indirecte(s) avec les cellules en cours d'initiation pathologique.

[0020] Plus particulièrement, dans un mode de mise en oeuvre de l'invention, la banque utilisée comprend en outre des acides nucléiques spécifiques de gènes dont le niveau d'expression est modifié dans une cellule

sanguine provenant d'un organisme présentant une situation pathologique.

[0021] L'invention est basée notamment sur une méthode originale, l'analyse qualitative des différences liées à la présence d'insertions ou de délétions (épissages alternatifs) dans des régions essentielles pour la fonction des produits des gènes. Ces insertions et délétions sont précisément régulées et sont caractéristiques des états physiologiques et physiopathologiques (notamment prolifératifs et différenciés) des cellules de l'organisme. Ce niveau de régulation est affecté au cours de l'initiation, du maintien et du développement d'un grand nombre de pathologies. Dans un mode préféré, l'invention réside donc aussi dans l'application d'une technologie génomique destinée à analyser systématiquement ces dérégulations à la mise au point de diagnostics prédictifs. L'invention permet ainsi d'identifier les gènes dérégulés dans des cellules circulantes lors d'événements pathologiques, et d'utiliser ces événements génétiques qualitatifs dans des tests diagnostiques, prédictifs ou de détection d'événements pathologiques, qui contribuent à la maîtrise globale des dépenses de santé.

[0022] Dans la perspective d'identifier des marqueurs d'expression génétique spécifiquement présents dans les cellules sanguines d'un organisme présentant une pathologie, par exemple à un stade trop précoce pour pouvoir être diagnostiquée par examens cliniques ou tests diagnostics classiques, la présente invention propose avantageusement d'identifier des altérations post-transcriptionnelles. En effet ces altérations sont principalement la conséquence de la modification de la régulation d'une étape-clef de l'expression génique : l'épissage. Les variations d'épissage modifient de façon qualitative les ARN en incluant ou en excluant de ceux-ci des exons ou introns dont la présence ou l'absence liée à une situation physiopathologique donnée peut fournir la base pour un diagnostic. Ce diagnostic peut être basé sur l'utilisation de PCR ou d'hybridations qui permettent de détecter spécifiquement la séquence épissée de façon différentielle entre les deux situations. Souvent les variations d'épissage, par utilisation d'exon(s) alternatif(s) ou par rétention d'intron(s) dans un ARN messager affectent la séquence de la protéine correspondante. Ces différences dans l'enchaînement d'acides aminés permettent d'envisager un diagnostic basé sur l'utilisation d'anticorps qui reconnaissent spécifiquement la séquence protéique alternative.

[0023] Comme indiqué ci-dessus, le procédé de l'invention repose notamment sur l'utilisation de cellules circulantes comme matériel biologique. Plus particulièrement, il s'agit de cellules sanguines, et de préférence de cellules nucléées. On peut citer notamment les lymphocytes, macrophages, monocytes, cellules dendritiques, etc. Ces cellules peuvent être prélevées chez un sujet par toute technique connue de l'homme du métier, cytophérèse, gradients Ficoll, préparation de cellules mononucléées du sang périphérique, etc. Pour la mise

en oeuvre de la présente invention, les différentes populations de cellules sanguines peuvent être séparées les unes des autres, pour n'utiliser qu'un type particulier, présentant une signature génomique spécifique. Toutefois, le test de l'invention peut également être réalisé sur un échantillon biologique comprenant de cellules sanguines non séparées. Par ailleurs, les cellules circulantes peuvent également être (ou comprendre) des cellules tumorales, détachées du tissu pathologique, par exemple dans le cas de processus de métastases. Les acides nucléiques peuvent être préparés de l'échantillon selon toute technique connue de l'homme du métier (lyse cellulaire, extraction, isolement des ARN, etc.). En outre, ces acides nucléiques sont préférentiellement traités préalablement à l'étape d'hybridation, par exemple pour produire de ADNc, pour amplifier ces acides nucléiques, pour les marquer, etc. A cet égard, le marquage peut être par exemple radioactif, enzymatique, fluorescent, colorimétrique, ou de toute autre nature. Typiquement, le procédé de l'invention comprend le prélevement d'un échantillon biologique sanguin, le traitement des cellules sanguines pour libérer les acides nucléiques, l'amplification des acides nucléiques (et, le cas échéant, leur transcription inverse), le marquage des acides nucléiques et leur hybridation sur la ou les banques.

[0024] Le procédé de l'invention peut être utilisé pour détecter la présence d'une pathologie (ou d'un événement pathologique), c'est-à-dire l'existence de mécanismes cellulaires caractéristiques d'une situation d'initiation ou de développement d'une pathologie, même si les symptômes cliniques ne sont pas encore apparents. Le procédé de l'invention peut à cet égard permettre également la détection *in vitro* du stade d'évolution d'une pathologie chez un sujet. Ainsi, les signatures génétiques des cellules évoluent en fonction du stade d'avancement de la pathologie, et il est possible de détecter, grâce à des banques spécifiques, l'évolution d'une pathologie. D'autre part, le procédé de l'invention permet aussi de détecter *in vitro* la localisation d'une pathologie chez un sujet, c'est-à-dire par exemple l'origine tissulaire du foyer pathologique.

[0025] Comme indiqué ci-dessus, le procédé de l'invention peut être mis en oeuvre pour détecter différents types de pathologies, notamment des pathologies associées à des dérégulations des voies de signalisations cellulaires. Il peut s'agir de pathologies liées au vieillissement, comme par exemple les maladies neurodégénératives, ou de toute autre pathologie impliquant notamment une prolifération cellulaire anormale (cancer, sténose, etc.).

[0026] Selon un mode particulier, l'invention concerne un procédé tel que défini ci-dessus pour la détection *in vitro* de la présence, du stade d'évolution et/ou de la localisation d'une pathologie neurodégénérative.

[0027] Selon un autre mode particulier, l'invention concerne un procédé tel que défini ci-dessus pour la détection *in vitro* de la présence, du stade d'évolution et/ou de la localisation d'une pathologie cancéreuse. Il peut s'agir de cancers variés, comme par exemple de tumeurs solides (hépatiques, pulmonaire, tête et cou, mélanome, foie, vessie, sein, etc.).

- [0028]** L'invention décrit également un procédé de détection *in vitro* de cellules sanguines caractéristiques d'un état pathologique, comprenant le prélèvement d'un échantillon de cellules sanguines chez un sujet et la détermination de la présence, dans cet échantillon, de celles sanguines présentant un profil génétique caractéristique d'une pathologie.
- [0029]** Comme décrit ci-dessus, l'invention repose en partie sur la constitution et l'utilisation de banques d'acides nucléiques caractéristiques d'un état pathologique.
- Dans un premier mode de réalisation, il s'agit de banques (ou de préparations) d'acides nucléiques, comprenant des acides nucléiques spécifiques de gènes dont le niveau d'expression est modifié dans une cellule sanguine provenant d'un organisme dans une situation pathologique.
- Selon un autre mode de réalisation, il s'agit de banques (ou de préparations) d'acides nucléiques comprenant des acides nucléiques spécifiques de formes d'épissages de gènes, caractéristiques d'une cellule sanguine provenant d'un organisme dans une situation pathologique.
- Les préparations et banques de l'invention peuvent être déposées sur des supports, affinées et mélangées, comme il sera décrit plus en détails dans la suite du texte.
- L'invention décrit encore des méthodes pour la préparation de telles banques. En particulier, ces méthodes comprennent (i) l'obtention d'une première préparation d'acides nucléiques à partir d'une cellule sanguine isolée d'un organisme présentant une pathologie neurodégénérative ou une tumeur solide, (ii) l'obtention d'une préparation d'acides nucléiques de référence à partir d'une cellule sanguine isolée d'un organisme ne présentant pas ladite pathologie, (iii) une étape d'hybridation entre ladite première préparation et la préparation de référence, et (iv) la récupération, à partir des hybrides formés, de clones d'acides nucléiques spécifiques de formes d'épissages de gènes caractéristiques de la cellule sanguine provenant de l'organisme en situation de pathologie.
- L'invention décrit également des procédés de préparation de banques d'acides nucléiques caractéristiques du stade d'évolution d'une pathologie, comprenant (i) l'obtention d'une première préparation d'acides nucléiques à partir d'une cellule sanguine isolée d'un organisme présentant une pathologie neurodégénérative ou une tumeur solide à un stade d'évolution déterminé, (ii) l'obtention d'une préparation d'acides nucléiques de référence à partir d'une cellule sanguine isolée d'un organisme présentant ladite pathologie à un stade d'évolution différent, (iii) une étape d'hybridation entre ladite première préparation et la préparation de référence, et (iv) la récupération, à partir des hybrides formés, de clones d'acides nucléiques spécifiques de formes d'épissages de gènes caractéristiques de la cellule sanguine provenant de l'organisme en situation de pathologie.

nes d'acides nucléiques spécifiques de formes d'épisages de gènes caractéristiques de la cellule sanguine provenant de l'organisme au stade d'évolution déterminé de la pathologie.

[0034] Comme il sera expliqué en détails plus loin, l'étape de récupération des clones peut comprendre soit la récupération des clones d'acides nucléiques non hybrides, soit la récupération, à partir des hybrides formés, de clones d'acides nucléiques spécifiques de formes d'épisages de gènes.

[0035] L'invention concerne également tout kit utilisable pour la mise en oeuvre d'un procédé tel que décrit ci-dessus comprenant une banque d'acides nucléiques comprenant des acides nucléiques spécifiques d'altérations d'expression génétique caractéristiques de cellules sanguines d'un organisme en situation pathologique.

Identification de marqueurs spécifiques des modifications transcriptionnelles et post-transcriptionnelles

[0036] Comme indiqué ci-dessus, l'invention décrit des procédés de préparation de banques d'acides nucléiques caractéristiques du stade d'évolution d'une pathologie, comprenant (i) l'obtention d'une première préparation d'acides nucléiques à partir d'une cellule sanguine isolée d'un organisme présentant une pathologie à un stade d'évolution déterminé, (ii) l'obtention d'une préparation d'acides nucléiques de référence à partir d'une cellule sanguine isolée d'un organisme présentant ladite pathologie à un stade d'évolution différent, (iii) une étape d'hybridation entre ladite première préparation et la préparation de référence, et (iv) la récupération des acides nucléiques caractéristiques de la cellule sanguine provenant de l'organisme au stade d'évolution déterminé de la pathologie.

[0037] Les méthodes de l'invention comprennent plus particulièrement la constitution de clones et de banques d'acides nucléiques à partir d'ARN(s) extraits de différentes pathologies, à différents stades de leur progression, et obtenues aussi bien à partir de tissus pathologiques que des cellules sanguines dont l'expression génétique a été affectée par ces tissus. L'obtention de ces clones et de ces banques se fait avantageusement par des techniques d'analyses différentielles de l'expression génétique. Les signatures différentielles obtenues sont donc spécifiques de différences entre tissu malade et tissu sain d'une part et cellules sanguines de malade et cellules sanguines de témoin sain d'autre part. Ces signatures peuvent donc être exprimées préférentiellement soit dans les échantillons pathologiques soit dans les échantillons contrôles.

[0038] Les populations d'acides nucléiques servant à l'obtention de clones ou à la constitution de banques sont par exemple des ARN (totaux ou messagers) des cellules extraits d'une situation pathologique et des ARN (totaux ou messagers) correspondant à une situa-

tion contrôle, ou des acides nucléiques dérivés des ces ARN totaux ou messagers (par transcription inverse, amplification, clonage dans des vecteurs, etc.). Ces acides nucléiques peuvent être préparés selon les techniques connues de l'homme du métier. Brièvement, ces techniques comprennent généralement une lyse des cellules, tissu ou échantillon, et l'isolement des ARNs par des techniques d'extraction. Il peut s'agir en particulier d'un traitement au moyen d'agents chaotropiques tels que le thiocyanate de guanidium (qui détruit les cellules et protège les ARN) suivi d'une extraction des ARN au moyen de solvants (phénol, chloroforme par exemple). De telles méthodes sont bien connues de l'homme du métier (voir Maniatis et al., Chomczynski et al., Anal. Biochem. 162 (1987) 156), et peuvent être aisément pratiquées en utilisant des kits disponibles dans le commerce tels que par exemple le kit US73750 (Amersham) pour les ARN totaux. Il n'est pas nécessaire que les ARN utilisés soient parfaitement purs, et notamment il n'est pas gênant que des traces d'ADN génomique ou d'autres composants cellulaires (protéine, etc.) subsistent dans les préparations, dès lors qu'ils n'affectent pas significativement la stabilité des ARNs. En outre, de manière facultative, il est possible d'utiliser non pas des préparations d'ARN totaux mais des préparations d'ARN messagers. Ceux-ci peuvent être isolés, soit directement à partir de l'échantillon biologique soit à partir des ARN totaux, au moyen de séquences polyT, selon les méthodes classiques. L'obtention d'ARN messagers peut à cet égard être réalisée au moyen de kits commerciaux tels que par exemple le kit US72700 (Amersham). Les ARN peuvent également être obtenus directement à partir de banques ou autres échantillons préparés à l'avance et/ou accessibles dans des collections, conservés dans des conditions appropriées.

[0039] L'hybridation peut être réalisée dans différentes conditions, qui peuvent être ajustées par l'homme du métier. De préférence, on utilise pour l'hybridation un excès de la population d'acides nucléiques dérivés de la situation de dérégulation par rapport à la population d'acides nucléiques dérivés de la situation de contrôle.

[0040] A partir du produit de la réaction d'hybridation, deux types principaux d'approches peuvent être utilisés pour isoler les clones caractéristiques de dérégulations (pathologiques) selon l'invention. La première, purement quantitative, permet de générer une préparation d'acides nucléiques regroupant l'ensemble (ou une partie significative) des clones résultant d'une différence de niveau d'expression entre les deux situations. De tels clones (et banques) sont obtenus selon les techniques connues d'hybridation soustractive, consistant essentiellement à éliminer les hybrides formés lors de l'étape d'hybridation, pour ne conserver que les clones non hybrides, caractéristiques de la situation de dérégulation par rapport à la situation de contrôle choisie.

[0041] Dans un mode préféré de mise en oeuvre, on utilise cependant un procédé qualitatif, qui permet de générer une préparation d'acides nucléiques regrou-

pant l'ensemble (ou une partie importante) des clones résultant d'altérations génétiques fonctionnelles caractéristiques de la situation de dérégulation par rapport à la situation de contrôle choisie. Plus particulièrement, une telle banque qualitative comprend non pas l'ensemble des clones dont l'expression est modifiée, mais par exemple les clones correspondant à des épissages ou à des délétions différentielles entre les deux situations. Compte tenu du rôle des épissages alternatifs dans les voies de régulation et de transformation cellulaires, de telles préparations (et banques) comportent avantageusement des clones ayant une valeur fonctionnelle importante, et donc susceptibles de refléter des modifications génétiques impliquées dans la situation de dérégulation. De tels clones permettent donc de constituer des banques plus prédictives et de générer des marqueurs génétiques plus représentatifs.

La constitution de telles banques qualitatives peut être réalisée par isolement, à partir des hybrides formés lors de l'étape d'hybridation, des régions d'acides nucléiques correspondant à des épissages différentiels ou à des délétions. Selon les méthodes employées, ces régions correspondent soit aux régions non appariées, soit aux régions appariées.

[0042] Ces deux approches sont décrites plus en détails dans ce qui suit.

Production et utilisation de banques différentielles quantitatives

[0043] Dans un premier mode de mise en oeuvre, on utilise donc dans la présente invention une banque différentielle quantitative, c'est à dire une banque comprenant des clones d'acides nucléiques correspondant à des gènes dont le niveau d'expression est modifié dans des cellules en situation pathologique par rapport à une situation contrôle. De telles banques peuvent être issues par exemple d'analyses différentielles quantitatives, et regrouper les séquences dont l'expression est augmentée ou diminuée lors de phénomènes de dérégulation cellulaire. Les méthodologies pour établir ce type de banque sont connues de l'homme de métier et peuvent être regroupées dans les catégories suivantes :

Soustraction électronique à partir de Séquençage à Haut Flux

[0044] Ce procédé est basé sur le séquençage aléatoire d'un certain nombre de cDNAs. Un moteur de recherche informatique peut ensuite être utilisé pour effectuer une soustraction entre-deux situations analysées.

« Serial Analysis of Gène Expression (SAGE) »

[0045] Ce procédé est basé sur la reconnaissance d'une signature associée à chaque cDNA en utilisant

des enzymes de restriction et des oligonucléotides adaptateurs. Cette étiquette correspond à une partie de la séquence du cDNA (longue de 10 nucléotides afin d'identifier de façon non ambiguë le cDNA correspondant). Ces étiquettes sont ensuite assemblées pour être séquencées puis analysées (Velculescu et coll., Science, 1995, 270 :484-487). Cette approche représente donc un raccourci vis-à-vis du séquençage systématique.

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« Nucleic Acid Arrays »

[0046] Cette méthode repose sur le dépôt d'acides nucléiques (oligonucléotides, fragments PCR, cDNAs) sur supports solides (membranes, plaques de verre, bio-puces) à plus ou moins haute densité. Des sondes provenant d'ARN messagers d'échantillons sains ou pathologiques sont ensuite utilisées pour hybridation afin d'identifier les messagers qui sont ou bien sur-exprimés ou bien réprimés.

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« Differential Display »

[0047] Cette technique utilise une amorce oligo-dT et des amorces aléatoires pour réaliser des réactions PCR sur des populations d'ADNc. Les produits de PCR sont alors comparés sur des gels très résolutifs. Les fragments exprimés de façon différentielle sont ensuite isolés et leur présence est confirmée par Northern-blots avant séquençage.

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Plusieurs variantes de cette technologie ont été développées (Prashar et Weissman, PNAS, 1996, 93 : 659-663). Ces variantes diffèrent par leur amores et par le choix des enzymes de restriction et des adaptateurs utilisés. Comme la technologie SAGE, elles s'adressent aux extrémités 3' des cDNAs. Plusieurs kits sont également disponibles sur le marché afin de rendre accessible cette approche.

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Clonage par soustraction

[0048] Cette technique est basée sur l'élimination de cDNAs communs à deux échantillons que l'on désire comparer. Ainsi, différents kits de soustraction dans lesquels le cDNA 'tester' est hybridé à un excès de cDNA 'driver' sont proposés (Clontech). Le produit final est constitué d'un pool de fragments amplifiés par PCR dérivé des cDNAs exprimés de façon différentielle, qui peut être cloné dans un vecteur approprié pour analyse ultérieure. La technologie RDA (Representational Difference Analysis) est également basée sur ce principe de soustraction (Lisitsyn et coll., Science, 1993, 259 : 946-951).

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[0049] La mise en oeuvre de ces techniques d'analyses différentielles permet donc de générer des clones et des banques quantitatifs, c'est-à-dire regroupant l'ensemble des séquences dont l'expression est augmentée ou diminuée lors de phénomènes de dérégulation

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(s) cellulaire(s) impliqués dans des pathologies.

Production et utilisation de banques qualitatives différentielles

[0050] Dans un autre mode de mise en oeuvre, on utilise avantageusement dans la présente invention une banque différentielle qualitative, c'est à dire une banque comprenant des clones d'acides nucléiques dont une partie au moins de la séquence correspond à la séquence de gènes épissés différemment entre des cellules correspondant à une situation pathologique et une situation contrôle. Ce type de banque regroupe donc les séquences épissées de manière différentielle lors de processus de dérégulation pathologique.

[0051] L'utilisation de ce type de banque est particulièrement avantageuse. En effet, les différentes voies de signalisation qui sont altérées dans nombre de pathologies, comme les cancers et les maladies neurodégénératives par exemple, impliquent des gènes et donc des ARNm dont l'expression est régulée par épissage alternatif. De plus, un nombre croissant d'exemples fournis par la littérature montre que des formes d'ARN spécifiquement observées en pathologie sont le résultat d'une altération de l'épissage. En relation avec l'originalité de l'invention, il faut souligner aussi que l'état d'activation des différents types cellulaires qui participent à la réponse immunitaire est régulé par des cascades de signalisation dont les acteurs sont régulés par épissage.

[0052] Ainsi, la démence Alzheimer, la maladie de Huntington, la maladie de Parkinson sont autant d'exemples de maladies ayant un réel impact économique et présentant une composante neurodégénérative. Même si la description des symptômes cliniques et l'identification de quelques gènes de susceptibilité ont permis de faire des pas significatifs dans la connaissance de ces maladies, les bases moléculaires soutenant le développement de ces maladies sont toujours très obscures. L'élucidation des cascades de signalisation, dérégulées dans ces états pathologiques, conduira d'une manière indubitable à la découverte de cibles propices à l'intervention diagnostique et thérapeutique. La littérature souligne l'importance des altérations des processus d'épissage des ARN.

- L' Amyotrophie Spinale est l'une des maladies génétiques les plus fréquentes. Deux gènes SMN1 et SMN2 codent pour des protéines identiques, la perte des deux allèles SMN1 et une altération de l'épissage du gène SMN2 concourent au développement de la maladie (Lorson et coll. Proc. Natl. Acad. Sci. USA 1999, 96: 6307-6311).
- Dans des biopsies de patients atteints de démence Alzheimer , il a été détecté des altérations spécifiques de l'épissage du gène de présinilline PS1 (Isoe-Wada et coll. Eur. J. Neurol., 1999 : 163-167)
- Le transporteur au glutamate est d'une importance majeure dans des maladies neurodégénératives

comme la Sclérose Amyotrophie Latérale ou l'épilepsie, par exemple. Des altérations de l'épissage de ce transporteur en affectent la fonctionnalité (Meyer et coll . Neurosci. Lett, 1998. 241 : 68-70).

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[0053] De nombreux exemples d'inactivation d'activité anti-oncogène résultant d'épissages alternatifs des messagers correspondant sont aujourd'hui connus :

- 10 - Dans les carcinomes pulmonaires à petites cellules, le gène de la protéine p130 qui appartient à la famille RB (protéine du rэтinoblastome) est muté au niveau d'un site consensus d'épissage. La conséquence de cette mutation est l'élimination de l'exon 2 qui a pour résultat une absence de synthèse de la protéine en raison de la présence d'un codon « stop » précoce. Cette observation a été la première à souligner l'importance des membres de la famille RB dans la tumorigénèse.
- 15 - Dans les cancers de la tête et du cou, l'un des mécanismes d'inactivation de p53 implique une mutation dans un site consensus d'épissage.
- Dans d'autres types de cancers du poumon, le gène de la protéine p16/INK4A, protéine qui est un inhibiteur des kinases cyclines-dépendantes CDK4 et CDK6 est muté au niveau d'un site donneur d'épissage. Le résultat de cette mutation est la production d'une protéine tronquée à demi-vie courte. Or la protéine p16 s'associe normalement à CDK4 et CDK6, empêchant leur association aux cyclines de type D et la phosphorylation en particulier de RB, ce qui a pour conséquence l'accumulation des formes hypophosphorylées, actives, de RB. En l'absence de p16, RB est inactivée par phosphorylation. Il est à noter que le locus p16 est en fait particulièrement complexe et qu'outre l'expression de p16, il permet celle de p19 par épissage alternatif. La protéine p19 qui n'a aucun acide aminé commun avec la protéine p16, peut s'associer au proto-oncogène MDM2 et bloquer le cycle cellulaire en présence de p53, exerçant ainsi une fonction « suppresseur de tumeur ».
- 20 - WT1, anti-oncogène qui code pour un répresseur transcriptionnel dont les altérations sont à l'origine des tumeurs de Wilms, est transcrit en plusieurs ARN messagers engendrés par épissages alternatifs. Dans les cancers du sein, les proportions relatives des différents variants sont modifiées par rapport au tissu sain, fournissant des outils diagnostics et des pistes pour comprendre l'importance des différents domaines fonctionnels de WT1 dans la progression tumorale.
- 25 - Ce même phénomène de modification des rapports entre différentes formes d'ARN messagers et d'isoformes protéiques est retrouvé pour la neurofibrine NF1 dans les neurofibromes.
- Cette notion de modulation des phénomènes d'épissage signant la progression tumorale est éga-

lement renforcée par l'exemple de HDM2. Cinq épissages alternatifs de HDM2 sont en effet détectés dans les carcinomes ovariens et pancréatiques et, fait particulièrement intéressant, leur expression augmente selon le stade d'avancement tumoral.

- La LTBP, composant de la matrice extracellulaire de divers tissus intervenant dans la sécrétion et le stockage du TGF- β est aussi produite sous différentes isoformes. L'une d'elle, probablement moins sensible à la protéolyse, semble moduler l'activité biologique du TGF- β et pourrait être impliquée dans différents états physiopathologiques hépatiques.

[0054] Les réponses immunitaires cellulaires et humorales sont sous contrôle transcriptionnel. La littérature fournit des exemples nombreux d'isoformes natives produites par épissage alternatif impliquées dans ces réponses immunitaires.

- les récepteurs « éboueurs » des macrophages sont des glycoprotéines membranaires essentiels aux réponses physiologiques et pathologiques de ces cellules sanguines et leurs fonctions sont régulées par des isoformes générées par épissage (Gough et coll. J.Lipid Res ; 1998 ; 39 :531-543.)
- L'activation des lymphocytes T requiert la présence fonctionnelle de plusieurs récepteurs et protéines régulatrices. Boriello et Coll. (J. Immunol. 1995 ; 155 :5490-5497) ont rapporté la présence d'isoformes du co-facteur d'activation B7, à la suite d'épissage alternatif de ce gène, soulignant ainsi la grande plasticité de la réponse immunitaire apportée par ces variants d'épissage.

[0055] Tröster et al (J. Exp. Med. 180 (1994) 2059) rapporte par ailleurs l'identification d'une forme d'épissage du gène La/SS-B, sans toutefois établir sa corrélation à une pathologie.

[0056] Pour tenir compte de ces phénomènes et de cette complexité, et isoler ainsi des signatures spécifiques d'un état pathologique et présentes dans les cellules sanguines, le procédé de l'invention utilise avantageusement, comme marqueurs génétiques, des événements d'épissages caractéristiques de situations de dérégulation.

[0057] Pour ce faire, la présente invention utilise par exemple des banques d'acides nucléiques qualitatives différentielles produites selon la méthodologie « DATAS » décrite dans la demande de brevet internationale non publiée n° PCT/FR 99/00547. En particulier, de telles banques peuvent être préparées par hybridation entre la population d'acides nucléiques dérivée des cellules isolées de la circulation sanguine à partir de la situation pathologique et la population d'acides nucléiques dérivée des cellules circulantes de la situation de contrôle, et isolement, à partir des hybrides formés, des acides nucléiques correspondant à des épissages différentiels.

[0058] Dans cette approche, l'hybridation est préférentiellement réalisée en phase liquide. En outre, elle peut être effectuée dans tout dispositif approprié, tel que par exemple des tubes (Eppendorf, par exemple), des plaques, ou tout autre support adapté et couramment utilisé en Biologie Moléculaire. L'hybridation est avantageusement réalisée dans des volumes compris entre 10 et 1000 μ l, par exemple entre 10 et 500 μ l. Il est entendu que le dispositif utilisé et les volumes utilisés peuvent être aisément adaptés par l'homme du métier. Les quantités d'acides nucléiques utilisées pour l'hybridation sont également connues de l'homme du métier. En général, il est suffisant d'utiliser des microgrammes d'acides nucléiques, par exemple de l'ordre de 0,1 à 100 μ g. Par ailleurs, il est possible d'utiliser les acides nucléiques dans un rapport driver/tester variant de 50 à 0,02 environ, de préférence de 40 à 0,1. De manière plus particulièrement avantageuse, on préfère que ce rapport soit proche ou supérieur à 1, avantageusement

entre 1 environ et 10 environ. Il est bien entendu que ce rapport peut être adapté par l'homme du métier selon les conditions du procédé (quantités d'acides nucléiques disponibles, situations physiologiques, but poursuivi, etc.). Les autres paramètres de l'hybridation (temps, température, force ionique) sont également adaptables par l'homme du métier. De manière générale après dénaturation des "tester" et "driver" (par chauffage par exemple), l'hybridation est réalisée pendant environ 2 à 24 heures, à une température de 37°C environ (éventuellement soumise à des sauts de température), et dans des conditions standard de force ionique (pouvant varier de 0,1 à 5M NaCl par exemple). Il est connu que la force ionique est un des facteurs déterminant la stringence d'une hybridation, notamment dans le cas d'hybridation sur support solide.

Selon un mode de mise en oeuvre particulier de l'invention, l'hybridation est réalisée en émulsion phénolique, par exemple selon la technique PERT ("Phenol Emulsion DNA Reassociation Technique) décrite par Kohne D.E. et al. (Biochemistry, Vol. 16, N° 24, pp 5329-5341, 1977). Avantageusement, l'hybridation est réalisée en émulsion phénolique maintenue par thermocycles (sauts de température de 37°C environ à 60/65°C environ) et non par agitation, selon la technique décrite par Miller et Riblet (NAR 23 (1995) 2339).

[0059] Toute autre technique d'hybridation en phase liquide, de préférence en émulsion, peut être utilisée dans le cadre de la présente invention. Par ailleurs, l'hybridation peut également se faire avec l'un des partenaires immobilisé sur un support. Avantageusement, c'est le driver qui est immobilisé. Cela peut être réalisé notamment grâce à des amores biotinyliées ou par toute autre technique d'immobilisation connue de l'homme du métier.

[0060] A partir des populations d'acides nucléiques générées par hybridation, les marqueurs génétiques de l'invention (les clones caractéristiques des altérations génomiques qualitatives) peuvent être identifiés par

toute technique connue de l'homme du métier. Dans le cas des hétéroduplex ARN/ADN, ces régions se présentent essentiellement sous forme de régions d'ARN non-appariées (boucles d'ARN), et peuvent être identifiées et clonées par séparation des hétéroduplex et des acides nucléiques simple-brin (excès d'acide nucléique n'ayant pas réagi), digestion sélective des ARN double-brins (domaines engagés dans les hétéroduplex), puis séparation des ARN simple-brin résultant et des ADN simple-brins. Dans le cas des hétérotriplex, ces régions d'épissages différentiels se présentent essentiellement sous forme de régions d'ADN double-brin et peuvent être identifiées et clonées par traitement en présence d'enzymes appropriées telles qu'une enzyme permettant de digérer les ARN, puis une enzyme permettant de digérer les ADN simple-brin. Les acides nucléiques ainsi obtenus sont directement sous forme d'ADN double-brin et peut être clonés dans tout vecteur approprié.

[0061] Il est entendu que d'autres variantes et conditions précises pour l'isolement des acides nucléiques, les hybridations et l'obtention des clones qualitatifs sont indiquées dans la demande n° PCT/FR99/00547 non encore publiée.

[0062] Ces méthodologies permettent de générer des clones et des banques d'acides nucléiques correspondant à des marqueurs génétiques qualitatifs qui permettent de distinguer les cellules sanguines d'une situation saine de celles d'une situation pathologique. Comme indiqué dans la section expérimentale, ces préparations d'acides nucléiques représentent des marqueurs particulièrement utiles pour diagnostiquer à partir d'un prélèvement sanguin des maladies neurodégénératives et des cancers.

Diversité des banques

[0063] Les méthodologies décrites ci avant permettent donc de générer des ensembles de clones d'acides nucléiques caractéristiques des différences entre situations saine et pathologique. Chaque technique de préparation génère de nombreux clones, constituant des banques. Ces banques peuvent être mises en oeuvre telles quelles, déposées sur des supports ou modifiées par addition ou suppression de clones, regroupement de différentes banques, ajout de clones témoins, etc.

[0064] Les banques de l'invention peuvent comprendre par exemple de 10 à 50 000 clones, plus généralement de 10 à 10 000 clones, encore plus préférentiellement de 50 à 5000 clones. Les clones sont généralement déposés, de façon ordonnée, sur un ou plusieurs supports, de façon à faciliter l'analyse des résultats d'hybridation. Le support peut être-en-verre, nylon, plastique, fibre, etc., de manière générale tout support solide adapté à l'étalement d'acides nucléiques. Le dépôt des banques sur les supports peut être réalisé par des techniques conventionnelles connues de l'homme du métier, qui sont décrites par exemples dans la demande internationale PCT/FR99/00547.

[0065] Les banques utilisées peuvent comprendre à la fois des clones d'acides nucléiques correspondant à des gènes dont le niveau d'expression est modifié (marqueurs génétiques quantitatifs) et des clones d'acides nucléiques dont une partie au moins de la séquence correspond à des exons ou à des introns épissés de façon différentielle entre une situation saine et une situation pathologique (marqueurs génétiques qualitatifs). Ainsi, les marqueurs génétiques peuvent être générés selon des approches différentes, puis mélangés pour obtenir une réponse aussi prédictive que possible. Il est également possible de réunir au sein d'une même banque, sur un même support, des marqueurs de l'expression génétique exprimés spécifiquement dans les cellules présentes dans la circulation sanguine lors de pathologies différentes. L'hybridation d'une telle banque permet donc un suivi du développement de plusieurs pathologies à partir d'un même prélèvement sanguin. Un objet de la présente invention réside donc également dans une préparation d'acides nucléiques comprenant des marqueurs génétiques qualitatifs et quantitatifs caractéristiques de dérégulation(s) cellulaire(s) présentes dans les cellules de la circulation sanguine et symptomatiques de pathologies. Un objet particulier réside dans une banque comprenant des marqueurs génétiques caractéristiques de différentes situations de dérégulation. L'invention a aussi pour objet tout support solide sur lequel au moins deux banques d'acides nucléiques caractéristiques de deux situations de dérégulation ont été déposées. A cet égard, l'invention concerne encore un procédé de préparation d'une puce à ADN utilisable pour le diagnostic de pathologies, comprenant le dépôt, sur un support solide, d'une ou plusieurs préparations d'acides nucléiques caractéristiques de situation(s) de dérégulation.

[0066] Par ailleurs, selon un mode préféré de mise en œuvre, on utilise des banques d'acides nucléiques affinées par sélection des clones au fur et à mesure de l'utilisation, en fonction de leur implication effective dans différentes pathologies ou différents stades d'une même pathologie. Les banques initiales peuvent en effet comprendre par exemple l'ensemble des clones caractéristiques des événements génétiques d'une situation de dérégulation consécutive à l'établissement d'une pathologie. Puis, la mise en œuvre du procédé de diagnostic de l'invention permet d'observer que certains des clones hybrident avec des sondes issues de stades précis et intermédiaires du développement de la pathologie. Ces clones peuvent donc être identifiés comme révélateurs de stades précoces et peuvent fournir un outil diagnostic très puissant capable d'anticiper tout autre critère d'examen clinique, tout-autre-outil-diagnostic.

[0067] De manière plus spécifique, la présente invention décrit à présent l'identification et la caractérisation de tels clones, utilisables comme marqueurs génétiques de la présence et de l'évolution de pathologies.

[0068] L'une des applications majeures de l'identification et du clonage de ces marqueurs génétiques con-

cerne l'évaluation du potentiel hybride des ARN extraits de cellules sanguines d'un individu donné. Cette évaluation peut s'effectuer en hybrideant une sonde représentant les ARN messagers des cellules de cet individu avec une ou plusieurs banques de signatures caractéristiques de situation(s) pathologique(s), telles que décrites ci avant. Cette application est décrite plus en détails dans ce qui suit.

Méthodes d'analyse et de diagnostic des signatures de pathologies

[0069] L'invention permet de déterminer la présence de signatures spécifiques de différents stades de pathologies par hybridation d'un échantillon d'acides nucléiques de cellules présentes dans la circulation sanguine avec les marqueurs génétiques définis ci-dessus, le profil d'hybridation observé indiquant les dérégulations physiopathologiques de l'organisme dont est issu l'échantillon sanguin. Dans ce but, les marqueurs génétiques utilisés sont préférentiellement regroupés sous forme de banques, afin de fournir une réponse aussi prédictive que possible. L'un des avantages de la présente invention réside également dans le nombre important de marqueurs utilisés, qui rend l'information générée d'autant plus prédictive. Le caractère prédictif de l'information est en outre consolidé par la nature des marqueurs utilisés et préparés.

[0070] Un objet particulier de l'invention réside dans une méthode d'analyse du statut des cellules sanguines, comprenant au moins une étape d'hybridation entre a) un échantillon d'acides nucléiques de cellules sanguines et b) une banque d'acides nucléiques correspondant à des événements génétiques caractéristiques de situation(s) de dérégulation(s) de voie(s) de signalisation cellulaire, le profil d'hybridation indiquant les dérégulations physiopathologiques de l'organisme.

[0071] D'autres aspects et avantages de la présente invention apparaîtront à la lecture de la section expérimentale suivante, qui doit être considérée comme illustrative et non limitative.

SECTION EXPERIMENTALE

Exemple de maladies neurodégénératives : ALS

[0072] Les modèles animaux donnent accès à des échantillons biologiques qui permettent d'analyser différentes étapes du développement d'une pathologie et de comparer ces étapes à des témoins sains.

La sclérose amyotrophique latérale (SAL/ALS) est une maladie neurodégénérative, associée à différents types d'inclusions tels les corps de Lewy et caractérisée par une apoptose des motoneurones spinaux et corticaux dont l'issue fatale est parfois associée à une démence frontale. Des formes sporadiques, sans aucune mutation décrite, coexistent avec des formes familiales (FALS) associées à des mutations dans le gène SOD1

codant pour la superoxyde dismutase. Des souris transgéniques qui expriment le gène humain SOD1 portant l'une des mutations qui prévaut dans les FALS (mutation G93A) sont disponibles auprès de Jackson Laboratory, sous condition de prise d'une licence d'utilisation auprès de la NorthWestern University. L'apparition des symptômes d'ALS liés à la mutation G93A dans SOD1 n'est pas la conséquence d'une réduction de l'activité superoxyde dismutase mais d'un gain de fonction qui augmente la capacité de l'enzyme à générer des radicaux libres. Ce modèle reproduit en 120 jours l'issue fatale de la maladie avec des symptômes comparables à ceux de la maladie humaine. Ce modèle permet d'avoir accès à des échantillons cérébraux, spinaux et du sang périphérique.

Identification de formes d'épissage spécifique du modèle d'ALS :

[0073] La société ExonHit Therapeutics développe une approche originale de criblage différentiel qualitatif basée sur la technologie DATAS (Differential Analysis of Transcripts Alternatively Spliced). Cette technologie fait l'objet d'une demande de brevet en Europe et aux Etats-Unis. Les séquences identifiées par DATAS peuvent dériver d'exons alternatifs ou de rétention d'introns dans une des deux situations physiopathologiques comparées. Les données obtenues caractérisent donc des modifications de l'expression de séquences d'ARN qui affectent des domaines fonctionnels de protéines. L'analyse qualitative différentielle est effectuée sur des échantillons d'animaux transgéniques et des contrôles syngéniques âgés de 60 et 100 jours. 60 jours correspondent à un stade qui précède de peu les premiers symptômes, mais qui est déjà caractérisé au niveau cébral par des changements dans la physiologie cellulaire, dont notamment une altération du métabolisme mitochondrial. A 100 jours, 50% des motoneurones corticaux et spinaux sont morts et un processus actif d'apoptose neuronale est engagé parallèlement à une activation astrocytaire.

L'analyse qualitative différentielle est donc effectuée :

- à partir d'ARN extraits d'échantillons de cerveau et de moelle épinière sans isolement préalable des neurones afin de prendre en compte un maximum d'événements d'épissages alternatifs liés au développement de la pathologie.
- à partir de sang total périphérique ou de fractions cellulaires sanguines Les séquences identifiées par DATAS correspondent à des introns et/ou à des exons dont les expressions différentielles par épissage entre les situations pathologiques et la situation saine sont validées par PCR.

La comparaison de ces séquences avec les banques de données permet de classifier les informations obtenues et de proposer une sélection raisonnée des deux séquences dont l'étude est à poursui-

vre.

Caractérisation ultérieure des séquences obtenues :

[0074] Les séquences validées par PCR peuvent être recherchées dans des modèles complémentaires impliquant des processus de neurodégénération. Ainsi des ARN provenant d'un modèle d'ischémie cérébrale ou des ARN d'un modèle animal de maladie à prions peuvent être utilement étudiés afin de valider l'expression sélective de marqueurs d'ALS ou plus généralement de marqueurs de maladies neurodégénératives.

[0075] L'expression des formes d'épissage identifiées sera recherchée dans des échantillons humains représentatifs de différentes pathologies à composantes neurodégénératives :

- prélèvements sanguins de patients atteints de maladies neurodégénératives comme la maladie d'Alzheimer, le Parkinson etc..

Exemple de cancer : cancer des voies aériennes et digestives supérieures.

[0076] La transmission de transgènes à fait l'objet de nombreuses applications expérimentales dans le domaine de la cancérologie expérimentale. Ainsi utilise-t-on les allèles dominants de certains gènes cancérogènes pour obtenir des souris transgéniques, alors modèle expérimental pour l'étude du cancer.

Les cancers constituent un groupe hétérogène de maladies caractérisées chacune par un ensemble complexe d'altérations génétiques ayant pour conséquence une prolifération cellulaire anarchique et la dissémination de métastases. Si l'identification des altérations génétiques qui provoquent l'apparition et la progression des cancers apparaît aujourd'hui essentielle pour diagnostiquer et suivre l'évolution tumorale, c'est la perspective d'élaborer de nouveaux diagnostics plus précoce sur la base de cette connaissance qui rend compte de l'importance des enjeux de ces recherches pour l'industrie pharmaceutique. Les gènes dont les altérations peuvent conférer à une cellule des propriétés cancéreuses, sont nombreux. Ils jouent des rôles essentiels non seulement au cours du développement mais aussi tout au long de la vie cellulaire. Ils interviennent ainsi pour assurer des fonctions aussi essentielles que la prolifération, la différenciation, la réparation de l'ADN ou la survie cellulaire. Ceux dont les altérations conduisent à la production de protéines qui activent anormalement le cycle cellulaire sont appelés des oncogènes. On trouve par exemple dans cette catégorie les gènes cellulaires myc et ras. Les anti-oncogènes ont au contraire pour fonction normale de freiner le cycle cellulaire. L'inhibition de leur activité met la cellule sous la seule dépendance des gènes à effet prolifératrice et favorise donc la progression tumorale. On trouve dans cette catégorie les gènes RB (rétinoblastome) et P53. A coté des on-

cogènes et anti-oncogènes, les gènes modulant la mort cellulaire programmée ou apoptose apparaissent comme des acteurs importants de la cancérogenèse. Assimilable à un processus de différenciation cellulaire physiologique, la mort cellulaire programmée est contrôlée génétiquement. Le fait qu'une cellule ait perdu la capacité d'enclencher cette différenciation terminale qui en permet l'élimination, la met en effet en situation de survie anormale et peut favoriser l'émergence d'un clone cellulaire transformé. C'est ce que l'on observe dans les lymphomes folliculaires humains où le gène bcl-2 se trouve sur exprimé en raison de la translocation survenue entre les chromosomes 14 et 18. Cette sur-expression d'un gène à activité anti-apoptotique favorise la survie anormalement longue de populations cellulaires au sein desquelles peuvent alors s'accumuler d'autres mutations transformantes. La mort cellulaire programmée ou apoptose est aujourd'hui reconnue comme un mécanisme essentiel à l'élimination de cellules devenues indésirables qu'il s'agisse de cellules infectées par un virus comme de cellules ayant accumulé des mutations les rendant non fonctionnelles ou anormalement proliférantes. Le niveau de complexité qui régit l'héméostasie cellulaire résulte non seulement du nombre important des acteurs impliqués mais aussi des rôles variés que chacun d'eux peut alternativement jouer en fonction du type cellulaire ou des conditions. L'anti-oncogène p53 ou le proto-oncogène cMyc peuvent par exemple jouer aussi un rôle important dans le contrôle de l'apoptose. Une telle complexité rend nécessaire la mise en œuvre d'approches assez globales pour analyser des modulations d'expression d'ensembles de gènes et suffisamment spécifiques pour, le plus rapidement possible identifier les altérations les plus pertinentes en regard du diagnostic, du suivi de la progression tumorale ou de l'identification de nouvelles cibles d'actions thérapeutiques.

En utilisant différents systèmes d'adressage (régions promotrices) il est possible d'obtenir d'une manière préférentielle l'expression du transgène dans un tissu particulier. On peut ainsi disposer de modèles de tumeurs se développant dans un environnement tissulaire spécifique. Ces différents modèles de tumeurs ciblées laissent entrevoir la possibilité de détecter des signatures spécifiques au niveau des cellules circulantes en fonction de la localisation de la tumeur.

Modèle de tumeur ciblée sur le foie.

[0077] Il existe chez la souris un modèle d'hépatocarcinome (HCC) lié à l'expression restreinte dans le foie des séquences précoces du virus SV40, codant pour les antigènes grand T et petit t (Dubois, N., Bennoun, M., Allemand, I., Molina, T., Grimber, G., Daudet-Monsac, M., Abelanet, R., and Briand, P. (1991) Time course development of differentiated hepatocarcinoma and lung metastasis in transgenic mice. J. Hepatol., 13, 227-239). Le transgène est sous le contrôle du promo-

teur du gène humain de l'antithrombine III ce qui entraîne une expression précoce et constante des antigènes viraux. De ce fait, la prolifération hépatocellulaire subit une perturbation en deux temps. L'indice de prolifération des hépatocytes transgéniques est proportionnellement supérieure à la normale pendant le développement hépatique (de la naissance à la 5^{ème} semaine), puis diminue sensiblement sans pour autant atteindre l'indice faible caractéristique de l'état quiescent d'un foie normal. Ces souris transgéniques développent de façon systématique des HCC différenciés conduisant à la mort de tous les animaux avant 7 mois. Malgré une dérégulation précoce de la prolifération des hépatocytes, l'hépatomégalie n'apparaît que tardivement. L'analyse des étapes préneoplasiques précédant l'apparition des HCC a permis de mettre en évidence un mécanisme compensateur par apoptose, maintenant une masse hépatique normale dans ce modèle (Allemand et al., 1995). Il est intéressant de noter que cette apoptose s'arrête au moment même où le foie normal entre dans un état quiescent. Au delà de ce point, il semble que l'homéostasie hépatique ne soit plus contrôlée. Une étude de systématique de la sensibilité à l'apoptose a révélé que les hépatocytes dérivés de ce modèle transgénique avaient acquis un caractère résistant à la mort cellulaire dépendante du système CD95/Fas (Rouquet N, Allemand I, Molina T, Bennoun M, Briand P and Joulin V. (1995) Fas-dependent apoptosis is impaired by SV40 T-antigen in transgenic liver. Oncogene, 11, 1061-1067 Rouquet N, Allemand I, Grimber G, Molina T, Briand P and Joulin V. (1996) Protection of hepatocytes from Fas-mediated apoptosis by a non-transforming SV40 T-antigen mutant. cell Death & Diff., 3, 91-96) par un mécanisme indépendant d'un épissage alternatif du récepteur CD95/Fas. Cependant, seule une analyse globale des modifications d'épissage peut rendre compte d'une altération de ce processus pour l'ensemble des partenaires moléculaires intervenant dans la voie de signalisation du récepteur CD95/Fas.

Ce modèle transgénique représente un outil idéal pour identifier 1) les modifications de l'expression génique accompagnant la transition préneoplasie vers néoplasie, que ce soient des gènes indispensables à la transformation (oncogènes) ou des gènes s'opposant à la progression tumorale (gènes apoptotiques) 2) des signatures circulantes du développement du cancer liées à l'échappement à partir de la tumeur de cellules tumorales 3) d'événements d'altération de l'expression génique dans les cellules du sang caractéristiques du développement du cancer.

Identification des signatures spécifiques

[0078] L'approche différentielle qualitative est menée sur des ARN extraits de foie et de cellules du sang de souris normales et de souris développant des hépatocarcinomes (HCC) liés à l'expression de l'antigène T de SV40 sous contrôle du promoteur de l'AntiThrombine III.

Les animaux contrôles et les animaux exprimant le transgène sont choisis à différents âges permettant d'avoir accès aux stades très précoces, préneoplastiques et néoplasiques caractérisés notamment dans ce modèle par une activation puis une inactivation de l'apoptose nécessaire à l'homéostase hépatique.

Utilisation des séquences identifiées

10 [0079] Les modulations de l'expression de ces séquences pourront être ensuite recherchées dans des biopsies de tumeurs humaines afin d'en élargir le champ d'application, en thérapie humaine et en diagnostic.

Ces ADNc sont utilisés pour suivre l'évolution tumorale dans ce modèle transgénique et dans une série

15 de modèles d'HCC murins produits par transgenèse (Bennoun M, Grimber G, Couton D, Seye A, Molina T, Briand P and Joulin V. (1998) The amino-terminal region of SV40 large T antigen is sufficient to induce hepatic tumours in mice Oncogene, 17, sous presse). Ainsi en utilisant les ADNc spécifiques détectés à différents stades très précoces dans les cellules sanguines, avant l'apparition des tumeurs, on peut prédire dans une population mixte de souris saines et de souris transgéniques, celles qui vont développer un cancer.

20 Des sondes nucléotidiques ou des amorces de PCR dérivés de ces ADNc spécifiques des tumeurs peuvent être utilisées pour rechercher l'expression, dans des biopsies de tumeurs humaines, des formes d'épissage identifiées et/ ou les ARN dont la quantité est altérée dans ce modèle.

25 De même, les sondes identifiées dans le sang des animaux à différents stades du développement tumoral peuvent également utilisées afin de détecter des signatures communes avec des prélèvements de sang de patients atteints de cancers.

30 Dans une stratégie qui utilise des banques d'ADNc obtenues selon les procédés de l'invention décrits plus haut, une sonde totale préparée à partir de prélèvement sanguins de patients atteints de cancer, peut également être utilisée pour la recherche de signatures communes aux différentes banques issues de modèles murins à différents stades du développement tumoral d'une part et à partie de biopsies de différents types tumoraux humains d'autre part. Ces hybridations sont réalisées selon les techniques connues de l'homme de métier (voir notamment les conditions d'hybridation décrites dans la demande PCT/FR99/00547).

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RECHERCHE DU DIAGNOSTIC PREDICTIF CHEZ L'HOMME

[0080] La méthodologie décrite dans ce chapitre pourra être mise en oeuvre indifféremment en utilisant les techniques d'analyse quantitative et qualitative décrites ci-avant. L'invention priviliege néanmoins l'utilisation et la recherche de marqueurs liés aux altérations qualitatives de l'expression des gènes en raison des

avantages décrits précédemment.

[0081] L'invention décrit l'identification et la constitution de banques de signatures caractéristiques de l'évolution d'une pathologie à partir de biopsies. Ainsi il est possible d'établir des banques de séquences d'ADNc représentatifs de l'évolution et de la localisation de ces pathologies. Il est donc possible de rechercher, à partir d'un échantillon sanguin, la présence de signatures ADNc identiques à celles présentes dans les banques. La présence de signatures communes souligne alors la présence d'acides nucléiques présentant les mêmes altérations que celles présentes dans les biopsies des pathologies suspectées, très vraisemblablement issues de cellules issues de tissus pathologiques.

[0082] La mise en oeuvre de cette recherche est notamment basée sur la confection de sondes à partir de cellules sanguines et l'hybridation de ces sondes avec des filtres qui regroupent différents marqueurs spécifiques de telle ou telle pathologie.

[0083] L'invention décrit la possibilité d'identifier des altérations de l'expression des gènes à partir de cellules sanguines et ceci à partir de modèles expérimentaux mimant tout ou partie d'une pathologie humaine (exemple modèle de souris ALS ou modèle de tumeur hépatique).

L'utilisation de sondes nucléiques dérivées de cellules sanguines de patients atteints ou non de la pathologie ciblée (maladie neurodégénérative, cancer etc..) permet de rechercher l'existence de signatures communes avec les banques prédictives expérimentales créées à partir des modèles pathologiques expérimentaux. L'apparition de signatures communes constitue un diagnostic d'un risque de développement d'une telle pathologie chez l'individu soumis au diagnostic.

[0084] L'invention permet également de procéder de la manière suivante :

- Des prélèvements sanguins de patients atteints ou non d'une pathologie ciblée sont mélangés afin de constituer un stock d'ARN représentatif des états pathologique et sain.
- Ces ARNs sont soumis à des analyses différentielles selon les techniques décrites dans l'invention et des banques d'ADNc caractéristiques des états pathologiques et sains sont constituées.
- Ces banques d'ADNc sont ensuite validées par hybridation à l'aide de sondes préparées à partir d'échantillons individuels de sang de patients ou de sujets sains.
- Les banques ainsi validées sont ensuite analysées par l'utilisation de sondes préparées à partir de prélèvements de sang d'une large population de sujets consultant un médecin pour des examens de routine. Ces banques constituent un outil diagnostique propre à l'invention. Ainsi un patient effectuant une mammographie pour dépistage d'un cancer du sein, peut être prélevé d'un petit échantillon de sang. Une sonde nucléique préparée à partir d'un

tel échantillon permet ensuite de rechercher très précocement des signes probables de l'évolution d'un cancer même en l'absence d'image à la radiographie.

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[0085] L'invention peut être utilisée sous forme de BioPuce. La BioPuce exploite les propriétés de la réaction d'hybridation par laquelle deux brins d'ADN dits complémentaires se lient l'un à l'autre d'une manière très spécifique. L'invention peut être utilisée en recherchant spécifiquement un ou plusieurs marqueurs ADN de la pathologie en utilisant une technique d'amplification de l'ADN à l'aide d'amorces oligonucléotidiques spécifiques de l'ADN à rechercher.

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Revendications

1. Procédé de détection in vitro de la présence d'une pathologie neurodégénérative ou d'une tumeur solide chez un sujet, comprenant (i) la préparation, à partir d'un échantillon de cellules sanguines du sujet, d'acides nucléiques comprenant des ARN ou des ADNc dérivés de ceux-ci et (ii) l'hybridation des acides nucléiques préparés avec au moins une banque d'acides nucléiques comprenant des acides nucléiques spécifiques de formes d'épissages de gènes caractéristiques d'une cellule sanguine isolée d'un organisme présentant une pathologie neurodégénérative ou une tumeur solide, le profil d'hybridation indiquant la présence de cellules sanguines caractéristiques de la pathologie dans l'échantillon.
2. Procédé selon la revendication 1, caractérisé en ce que la banque comprend en outre des acides nucléiques spécifiques de gènes dont le niveau d'expression est modifié dans une cellule sanguine isolée d'un organisme présentant une pathologie neurodégénérative ou une tumeur solide.
3. Procédé selon l'une des revendications 1 ou 2, caractérisé en ce que la ou les banques sont déposées sur un support.
4. Procédé selon l'une des revendications 1 à 3, caractérisé en ce que les acides nucléiques préparés à partir de l'échantillon sont des ARN totaux ou messagers, éventuellement amplifiés, ou des ADNc dérivés de ceux-ci.
5. Procédé selon la revendication 4, caractérisé en ce que les acides nucléiques sont marqués.
6. Procédé selon l'une quelconque des revendications précédentes, caractérisé en ce que les cellules sanguines sont des cellules nucléées.

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7. Procédé selon la revendication 6, **caractérisé en ce que** les cellules sanguines nucléées comprennent des lymphocytes, des macrophages, des monocytes et/ou des cellules dendritiques.
8. Procédé selon l'une des revendications précédentes, pour la détection *in vitro* de la présence de la sclérose amyotrophique latérale (ALS) chez un sujet.
9. Procédé selon l'une des revendications précédentes, pour la détection *in vitro* de la présence d'une tumeur solide sélectionnée parmi les tumeurs du foie, poumon, tête et cou, mélanome, vessie, sein et prostate chez un sujet.
10. Procédé selon l'une quelconque des revendications précédentes, pour la détection *in vitro* du stade d'évolution et/ou de la localisation d'une pathologie neurodégénérative,
11. Procédé selon l'une quelconque des revendications 1 à 9, pour la détection *in vitro* du stade d'évolution et/ou de la localisation d'une tumeur solide.
12. Procédé de préparation d'une banque d'acides nucléiques caractéristiques d'un état pathologique, **caractérisé en ce qu'il comprend** (i) l'obtention d'une première préparation d'acides nucléiques à partir d'une cellule sanguine isolée d'un organisme présentant une pathologie neurodégénérative ou une tumeur solide, (ii) l'obtention d'une préparation d'acides nucléiques de référence à partir d'une cellule sanguine isolée d'un organisme ne présentant pas ladite pathologie, (iii) une étape d'hybridation entre ladite première préparation et la préparation de référence, et (iv) la récupération, à partir des hybrides formés, de clones d'acides nucléiques spécifiques de formes d'épissages de gènes caractéristiques de la cellule sanguine provenant de l'organisme en situation de pathologie.
13. Procédé de préparation d'une banque d'acides nucléiques caractéristiques du stade d'évolution d'une pathologie, **caractérisé en ce qu'il comprend** (i) l'obtention d'une première préparation d'acides nucléiques à partir d'une cellule sanguine isolée d'un organisme présentant une pathologie neurodégénérative ou une tumeur solide à un stade d'évolution déterminé, (ii) l'obtention d'une préparation d'acides nucléiques de référence à partir d'une cellule sanguine isolée d'un organisme présentant ladite pathologie à un stade d'évolution différent, (iii) une étape d'hybridation entre ladite première préparation et la préparation de référence, et (iv) la récupération, à partir des hybrides formés, de clones d'acides nucléiques spécifiques de formes d'épissages de gènes caractéristiques de la cellule sanguine

provenant de l'organisme au stade d'évolution déterminé de la pathologie.

- 5 14. Procédé selon l'une des revendications 12 ou 13, **caractérisé en ce que** la banque est déposée sur un support.

Patentansprüche

- 10 1. Verfahren zum *in vitro* Nachweis des Vorliegens einer neurodegenerativen Krankheit oder eines festen Tumors bei einer Person, umfassend (i) Herstellung von Nucleinsäuren aus einer Probe an Blutzellen der Person, umfassend RNAs oder davon abstammende cDNAs, und (ii) Hybridisierung der hergestellten Nucleinsäuren mit wenigstens einer Bank an Nucleinsäuren, umfassend Nucleinsäuren, die für Spleißformen von charakteristischen Genen einer Blutzelle spezifisch sind, die aus einem eine neurodegenerative Krankheit oder einen festen Tumor aufweisenden Organismus isoliert ist, wobei das Hybridisierungsmuster auf das Vorliegen von Blutzellen in der Probe hinweist, die für die Krankheit charakteristisch sind.
- 15 2. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** die Bank außerdem Nucleinsäuren umfasst, die für Gene spezifisch sind, deren Expressionsniveau in einer Blutzelle verändert ist, die aus einem eine neurodegenerative Krankheit oder einen festen Tumor aufweisenden Organismus isoliert ist.
- 20 3. Verfahren nach einem der Ansprüche 1 oder 2, **dadurch gekennzeichnet, dass** die Bank oder die Banken auf einem Träger angeordnet sind.
- 25 4. Verfahren nach einem der Ansprüche 1 bis 3, **dadurch gekennzeichnet, dass** die aus einer Probe hergestellten Nucleinsäuren Gesamt-RNAs oder mRNAs, möglicherweise amplifiziert, oder davon abstammende cDNAs sind.
- 30 5. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** die Nucleinsäuren markiert sind.
- 35 6. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die Blutzellen Zellkerne enthalten.
- 40 7. Verfahren nach Anspruch 6, **dadurch gekennzeichnet, dass** die Zellkerne enthaltenden Blutzellen Lymphocyten, Makrophagen, Monocyten und/oder dendritische Zellen umfassen.
- 45 8. Verfahren nach einem der vorhergehenden Ansprüche zum *in vitro* Nachweis des Vorliegens der
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amyotrophen Lateralsklerose (ALS) bei einer Person.

9. Verfahren nach einem der vorhergehenden Ansprüche zum in vitro Nachweis des Vorliegens eines festen Tumors, ausgewählt aus Tumoren der Leber, Lunge, des Schädelns und Halses, Melanom, der Blase, Brust und Prostata bei einer Person.

10. Verfahren nach einem der vorhergehenden Ansprüche zum in vitro Nachweis des Entwicklungsstadiums und/oder der Lokalisierung einer neurodegenerativen Krankheit.

11. Verfahren nach einem der Ansprüche 1 bis 9 zum in vitro Nachweis des Entwicklungsstadiums und/oder der Lokalisierung eines festen Tumors.

12. Verfahren zur Herstellung einer Bank an Nucleinsäuren, die für einen Krankheitszustand charakteristisch sind, **dadurch gekennzeichnet, dass** das Verfahren umfasst (i) Gewinnung eines ersten Nucleinsäure-Präparates aus einer Blutzelle, die aus einem eine neurodegenerative Krankheit oder einen festen Tumor aufweisenden Organismus isoliert ist, (ii) Gewinnung eines Präparates aus Referenz-Nucleinsäuren aus einer Blutzelle, die aus einer besagte Krankheit nicht aufweisenden Organismus isoliert ist, (iii) einen Hybridisierungsschritt mit besagtem ersten Präparat und dem Referenz-Präparat, und (iv) und die Wiedergewinnung, aus den gebildeten Hybriden, der Nucleinsäure-Klone, die für Spleißformen von charakteristischen Genen der Blutzelle spezifisch sind, die vom Organismus in dem Krankheitszustand stammt.

13. Verfahren zur Herstellung einer Bank an Nucleinsäuren, die für das Entwicklungsstadium einer Krankheit charakteristisch sind, **dadurch gekennzeichnet, dass** das Verfahren umfasst (i) Gewinnung eines ersten Nucleinsäure-Präparates aus einer Blutzelle, die aus einem eine neurodegenerative Krankheit oder einen festen Tumor in einem bestimmten Entwicklungsstadium aufweisenden Organismus isoliert ist, (ii) Gewinnung eines Präparates aus Referenz-Nucleinsäuren aus einer Blutzelle, die aus einer besagte Krankheit in einem anderen Entwicklungsstadium aufweisenden Organismus isoliert ist, (iii) einen Hybridisierungsschritt mit besagtem ersten Präparat und dem Referenz-Präparat, und (iv) und die Wiedergewinnung, aus den gebildeten Hybriden, der Nucleinsäure-Klone, die für Spleißformen von charakteristischen Genen der Blutzelle spezifisch sind, die vom Organismus in dem bestimmten Entwicklungsstadium der Krankheit stammt.

14. Verfahren nach einem der Ansprüche 12 oder 13,

dadurch gekennzeichnet, dass die Bank auf einem Träger angeordnet ist.

5 Claims

1. A process for the in vitro detection of the presence of a neurodegenerative disease or of a solid tumor in a subject, comprising (i) preparing, from a sample of blood cells from the subject, nucleic acids comprising RNAs or cDNAs derived therefrom and (ii) hybridizing the nucleic acids so prepared with at least one nucleic acid library comprising nucleic acids specific for splicing forms of genes characteristic of a blood cell isolated from an organism presenting a neurodegenerative disease or a solid tumor, the hybridization profile indicating the presence of blood cells characteristic of the pathology in the sample.
2. Process according to claim 1, wherein the library further comprises nucleic acids specific for genes whose level of expression is modified in a blood cell isolated from an organism presenting a neurodegenerative disease or a solid tumor.
3. Process according to any one of claims 1 or 2, wherein the library(ies) is(are) deposited on a support.
4. Process according to any one of claims 1 to 3, wherein the nucleic acids prepared from the sample are total or messenger RNAs, optionally amplified, or cDNAs derived therefrom.
5. Process according to claim 4, wherein the nucleic acids are labelled.
6. Process according to any one of the preceding claims, wherein the blood cells are nucleated cells.
7. Process according to claim 6, wherein the nucleated blood cells comprise lymphocytes, macrophages, monocytes and/or dendritic cells.
8. Process according to any one of the preceding claims, for the in vitro detection of the presence of Amyotrophic Lateral Sclerosis (ALS) in a subject.
9. Process according to any one of the preceding claims, for the in vitro detection of the presence of a solid-tumor selected-from liver, lung, head-and-neck, melanoma, bladder, breast and prostate tumors in a subject.
10. Process according to any one of the preceding claims, for the in vitro detection of the stage of progression and/or the site of a neurodegenerative dis-

ease.

11. Process according to any one of claims 1 to 9, for
the in vitro detection of the stage of progression
and/or the site of a solid tumor. 5
12. A process of preparation of a nucleic acid library
characteristic of a pathological condition, wherein
said process comprises (i) obtaining a first nucleic
acid preparation from a blood cell isolated from an
organism presenting a neurodegenerative disease
or a solid tumor, (ii) obtaining a reference nucleic
acid preparation from a blood cell isolated from an
organism not presenting said pathology, (iii) a hy-
bridization step between said first preparation and
said reference preparation, and (iv) recovering,
from the hybrids formed, nucleic acid clones specif-
ic for splicing forms of genes characteristic of the
blood cell from the organism in pathological condi-
tion. 10 15 20
13. A process of preparation of a nucleic acid library
characteristic of the stage of progression of a pa-
thology, wherein said process comprises (i) obtain-
ing a first nucleic acid preparation from a blood cell
isolated from an organism presenting a neurode-
generative disease or a solid tumor at a defined
stage of progression, (ii) obtaining a reference nu-
cleic acid preparation from a blood cell isolated from
an organism presenting said pathology at a different
stage of progression, (iii) a hybridization step be-
tween said first preparation and said reference
preparation, and (iv) recovering, from the hybrids
formed, nucleic acid clones specific for splicing
forms of genes characteristic of the blood cell from
the organism at the defined stage of progression of
the pathology. 25 30 35
14. Process according to any one of claims 12 or 13,
wherein the library is deposited on a support. 40

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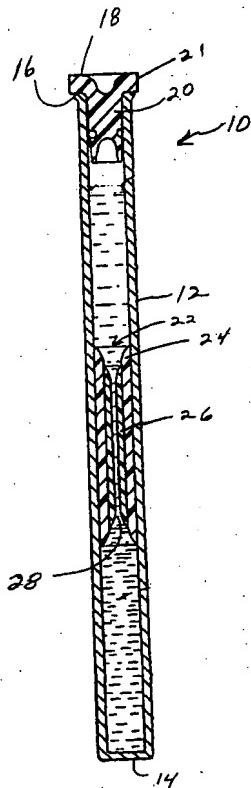
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(54) Method for separating cells from a sample

(57) Rare cells are separated from a sample fluid by a positive selection or negative selection antibody by centrifuging in a tube containing a harvesting float. The harvesting float has an axial passage and a density to settle in the sample fluid and expand the layer of the target component. The antibody is preferably coupled to a particulate carrier, such as a microbead, to attach either the target component or a contaminating component to the particulate carrier. In the positive separation, the particulate carrier is recovered in the axial passage of the float.

FIG. 2



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Description**Field of the Invention**

[0001] The present invention is directed to a method of separating a target component and particularly target cells from a sample. More particularly, the invention is directed to a method of separating target cells from a biological sample by positive or negative separation and centrifugation.

Background of the Invention

[0002] Numerous methods are known in the art for separating various constituents from biological fluids, and particularly blood samples. For example, the analysis of blood components typically involves the centrifugation of anti-coagulated whole blood to separate the cells from plasma and to separate the various cells into layers according to the density of the cells. After centrifugation, the plasma fraction is removed from the sample. Blood collection is often performed in an evacuated tube and then cell separation is achieved by centrifugation of the collection tube. The tube can contain a separator body that is made of a plastic material with a specific gravity that will enable the separator to settle during the centrifugation step onto the top of the formed component layer in the blood sample. The separator prevents mixing of the formed and unformed component fractions in the centrifuged blood sample. The separator also stabilizes the centrifuged layers for separation and analysis.

[0003] Another method of recovering cells from a blood sample uses a hollow insert placed in the centrifuge tube that contains the sample prior to centrifugation. The insert is made of a transparent plastic material and fits within the centrifuge tube. The insert slides within the tube when centrifuged to force the sample into the bore of the insert. The cells to be harvested from the sample collect in the bore of the insert thereby forming layers of constituents that separate according to the specific gravity of the constituents. The bore of the insert has a dimension to cause the layers to elongate in comparison to the thickness of the layer that would otherwise form in the tube without the insert. The resulting layers in the bore can be differentiated and removed from the bore using a hypodermic syringe or other cannula. An example of this process and device are disclosed in U.S. Patent No. 5,393,674 to Levine et al.

[0004] Another method and apparatus for separating constituents from a sample are disclosed in U.S. Patent No. 5,707,876 to Levine. This device uses one or more boundary makers that are placed in the tube before centrifugation. The markers slide within the tube when centrifuged and identify boundaries of the constituent layers that gravimetrically separate during centrifugation. A cannula is inserted into the tube through an elastomeric cap for injecting a liquid or gas into the tube. The injected

material displaces the centrifuged sample and the boundary markers to one end of the tube to express the centrifuged sample from the tube.

[0005] Other methods of separating components from a biological sample use paramagnetic microbeads having an antigen coupled thereto. The sample is mixed with the microbeads and incubated to bind the constituent to the microbead. The sample is then subjected to magnetic separation. An example of this type of method is disclosed in U.S. Patent No. 5,916,818 to Irsch et al.

[0006] These prior processes have been generally effective for their intended purpose. However, there is a continuing need in the industry for improved methods for separating cells from a biological sample.

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Summary of the Invention

[0007] The present invention is directed to a method for separating cells from a sample, and particularly a biological sample. Accordingly, a primary object of the invention is to provide a method for harvesting a specific constituent from a biological sample.

[0008] Another object of the invention is to provide a method for the separation of a specific constituent from a biological fluid in higher concentrations than can be obtained by prior methods.

[0009] A further object of the invention is to provide a method for harvesting selected cells from a biological fluid with low levels of contaminating constituents.

[0010] Still another object of the invention is to provide a method for harvesting rare cells from a biological fluid where the harvested rare cells are substantially free of mononuclear cells.

[0011] Another object of the invention is to provide a method for harvesting cells from a biological sample using a particulate carrier having a coating of an antibody having an affinity for a target cell in the sample.

[0012] A further object of the invention is to provide a method for harvesting a target constituent from a biological sample using microbeads coated with an antibody having an affinity for white blood cells.

[0013] Another object of the invention is to provide a method of separating a target component from a biological sample by centrifuging the sample in the presence of a float having an axial bore after combining the sample with a binding agent having an affinity for at least one component of the sample.

[0014] Still another object of the invention is to provide a method for harvesting a target component from a biological sample by centrifuging the sample in the presence of a particulate carrier having a positive or negative selectivity for the target component.

[0015] Another object of the invention is to provide a method of harvesting cells from a biological sample by mixing the sample with an amount of carrier particles containing an antibody having an affinity for white blood cells and where the particles have a density greater than the density of white blood cells for removing white blood

cells from the sample.

[0016] A further object of the invention is to provide a method of harvesting target cells from a biological sample by mixing the sample with an amount of carrier beads containing an antibody having an affinity for the target cells and the beads having a density less than the density of white blood cells for removing the target cells from the sample.

[0017] Still another object of the invention is to provide a method for separating target cells from a sample and detecting the target cells in a tube, where the target cells are separated by mixing the sample with carrier beads having an affinity for either the target cells or contaminating cells.

[0018] The objects and advantages of the invention are basically attained by providing a method of harvesting components from a sample material. The method comprises the steps of providing a sample material in a sampling container, the sampling container having a focusing device with a passage for receiving and elongating layers of sample components to be harvested from the sample, providing at least one antibody in the sampling receptacle, and mixing the antibody with the sample, wherein the antibody has an affinity for binding with at least one substance in the sample, and centrifuging the container and sample at sufficient G forces to separate components from the sample and to force a target component from the sample into the through passage.

[0019] The objects of the invention are further attained by providing a method of harvesting a target component from a whole blood sample. The method comprises the steps of providing a whole blood sample in a sampling tube, the sampling tube containing a float dimensioned to fit within the sampling tube and having a through passage for receiving and elongating layers of blood constituents to be harvested from the sample, mixing the sample with at least one particulate carrier containing an antibody having a binding affinity for a specific sample constituent, centrifuging the tube and sample at sufficient G forces to move the float toward one end of the tube and to force a target component from the sample into the through passage, and removing the target component from the through passage.

[0020] The objects of the invention are also attained by providing a method of harvesting a target component from a whole blood sample. The method comprises the steps of providing a whole blood sample in a sampling tube, the sampling tube containing a float dimensioned to fit within the sampling tube and having a through passage for receiving and elongating layers of blood constituents to be harvested from the sample, mixing the sample with an amount of first carrier beads having a coating of a first antibody that has a binding affinity for a target constituent in the sample and an amount of second carrier beads having a coating of the second antibody that has a binding affinity for white blood cells, centrifuging the tube and sample at sufficient G forces to move the float toward one end of the tube and to force

the first carrier beads and target constituent into the through passage, and removing the first carrier beads and target constituent from the through passage.

[0021] These objects, advantages and other salient features of the invention will become apparent from the in view of the annexed drawings and the following detailed description of the invention.

Brief Description of the Drawings

[0022] The following is a brief description of the drawings in which:

Figure 1 is a cross-sectional side view of the centrifuge tube in one embodiment of the invention showing a float member positioned in the tube;
 Figure 2 is a cross-sectional view of the tube of Figure 1 after centrifugation;
 Figure 3 is a perspective view of a centrifuge separation device in another embodiment of the invention;
 Figure 4 is a top view of the float of the embodiment of Figure 3; and
 Figure 5 is a side view in cross-section of the float taken along line 5-5 of Figure 4.

Detailed Description of the Preferred Embodiments

[0023] The present invention is directed to methods for harvesting a target component and particularly target cells from a biological sample. More particularly, the invention is directed to methods for harvesting rare cells from a biological sample with fewer contaminating cells present in the harvested rare cells.

[0024] In a preferred embodiment, the method of harvesting a target component utilizes at least one binding agent capable of binding with a component of the sample to assist in isolating or enhancing the target component during centrifugal separation. The binding agent can be an antibody selected to have an affinity for either the target component or one or more contaminating components. In preferred embodiments of the invention, the affinity binding agent, such as an antibody, is provided as a coating on a particulate carrier having a particle size and density that is compatible with the component being harvested to enhance separation and recovery of the target component from the sample. In the embodiments of the invention discussed below in greater detail, the particulate carrier can have a density lighter or heavier than the density of the contaminating constituent of the sample.

[0025] In further embodiments, the method is directed to harvesting rare cells and particularly tumor cells from biological samples and particularly anticoagulated whole blood samples. The method can be used to recover a variety of other cell types, such as stem cells and fetal cells, from blood samples.

[0026] The method of the invention in one embodi-

ment subjects the biological sample to centrifugal separation, where the sample is mixed with a particulate carrier having an antibody or other affinity binding agents bound to the surface of the particulate carrier. The centrifugation step preferably uses a centrifuge harvesting device. The antibody is preferably provided as a coating on the surface of the particulate carrier. The particulate carrier in preferred embodiments is an amount of microbeads made of a suitable nonreactive plastic resin. Examples of suitable plastic resins include polystyrene, polydivinylbenzene and polyvinylchloride.

[0027] The microbeads for use in the method of the invention can be produced by various methods as known in the art. In embodiments of the invention, the microbeads have a particle size ranging from about 0.05 microns to about 7 microns, and typically about 4 microns to about 5 microns.

[0028] The particulate carrier, such as the plastic microbeads, have a density that complements the various components of the sample to enable enrichment of the target component and particularly to enable enrichment of rare cells. In one embodiment, the particulate carrier includes an antibody having a binding affinity for the target component, such as tumor cells. In further embodiments, other affinity binding agents can be used. It was generally believed that after centrifugation, tumor cells are concentrated at the interface of the platelet/plasma region and above the denser majority white cells. The tumor cells harvested generally have a high concentration of contaminating mononuclear cells. It has now been found that tumor cells are not always concentrated at the interface between the cell layers and are difficult to recover without significant contamination from other interfering cells. In one embodiment of the invention, the particulate carrier has a density that is lower than the density of white blood cells so that the particulate carrier and the tumor cells that are bound to or captured on the carrier are concentrated above the layer of white blood cells.

[0029] In a first embodiment of the invention, the method of harvesting and enriching a target component is a positive selection process comprising the steps of contacting the sample fluid with a binding agent that is able to bind with the target component, and centrifuging the sample with a device capable of expanding constituent layers and enriching the target component. The target component can then be harvested and further processed to identify and culture the component by known methods. Examples of suitable identifying processes include flow cytometry and molecular nucleic acid amplification.

[0030] The centrifugation in one embodiment is carried out using a centrifuge device 10 as shown in Figures 1 and 2. Device 10 in the embodiment illustrated, includes a container, such as tube 12, that is preferably made of glass or other transparent material, such as plastic. Tube 12 has a closed bottom end 14 and an open top end 16. A stopper 18 is fitted in open top end

16 to close tube 12. Preferably, stopper 18 is made of a suitable elastomeric or rubber-like material that can be pierced by a cannula, needle or other piercing device. Stopper 18 has a substantially cylindrical body portion

5 20 having an outer dimension to form a snug friction fit in top end 16 of tube 12. A shoulder 21 extends radially outward from an upper end of stopper 18 to engage top end 16 of tube 12.

[0031] Tube 12 has a length and diameter suitable for 10 centrifuging a sample fluid. In one embodiment, tube 12 has a length of about 75 mm, an internal diameter of about 40 mm and a capacity of about 0.9 ml.

[0032] A float 22 is disposed in tube 12 as shown in 15 Figure 1. Float 22 is dimensioned to fit snugly in tube 12 and slide along the length of tube 12 during centrifuging of the sample. Float 22, in the embodiment illustrated, includes an outer sleeve 24 having a cylindrical shape complementing the inner surface of tube 12. Outer sleeve 24 is preferably made from a pliable material, such as a vinyl resin, that is able to deform slightly during centrifugation. Outer sleeve 24 can expand and contract in response to the centrifugal force so that float 22 is able to slide within tube 12. The pliable material returns to its original shape and dimensions at static conditions 20 so that outer sleeve 24 snugly contacts the inner surface of tube 12 and is able to slide within tube 12 under centrifugal force. A silicone lubricant can be applied to the inner surface of tube 12 to assist in the sliding movement of the float 22.

[0033] Float 22 includes an inner sleeve 26 having an 25 axial passage or bore 28 forming a through passage. Inner sleeve 26 is coupled to outer sleeve 24 by a bonding agent or other suitable method. Inner sleeve 26 is made of a rigid material that is dimensionally stable during centrifugation so substantially no distortion occurs during centrifugation. Axial bore 28 has a length and diameter suitable for expanding a fraction of the sample material during centrifugation. In one embodiment of the invention, axial bore 28 has an inner diameter of about

30 1.265 mm and a length of about 4.0 mm. Inner sleeve 26 is preferably made from a plastic such as polystyrene that does not interfere with the components of the sample. Float 22 is intended to be exemplary of a suitable centrifuge device capable of separating and expanding 35 a cell fraction. It will be understood that there are other devices that can be used during centrifugation to separate rare cell fractions. Suitable devices typically include a passage or a constricted area for elongating constituent layers during centrifugation to enable separation of the constituent layers.

[0034] Float 22 is dimensioned to fit in tube 12 and 40 slide within the tube 12 while centrifuging to settle between selected density layers of the sample fluid and force the target component into the axial bore 28 of float 22. Axial bore 28 of float 22 has an internal volume suitable to collect a substantial portion of the target component. The internal volume and diameter of the float effectively expand the layer of the target component. An

example of this type of cell harvesting device is disclosed in U.S. Patent No. 5,393,674 to Levine et al., which is hereby incorporated by reference in its entirety.

[0035] Float 22 is selected to have a density to complement the target component and the sample so that the target component collects in the axial bore 28 by enabling float 22 to settle at a predetermined point in the sample. In one embodiment of the invention, float 22 has a density to settle between the resulting plasma layer and the layer of red blood cells after centrifugation. In this embodiment, the rare cells that normally collect at the interface between the plasma and red blood cell layers collect in the axial bore 28 where they can be recovered.

[0036] The sample is centrifuged at a rate sufficient to separate the various constituents into layers. The centrifuge can be at a speed to produce a centrifugal force of about 400 G to about 800 G depending on the sample fluid. In embodiments, the centrifuge can produce a force of 1,000 G or more.

[0037] The method of the invention can be a positive selection or a negative selection for harvesting components, and particularly rare cells. In the positive selection method, a sample fluid is mixed with at least one antibody having an affinity for the target component. A negative selection mixes the sample with an antibody having an affinity for the contaminating cells, such as leukocytes and/or red blood cells. Suitable negative selection antibody reagents in the form of specialized conjugates and complexes are available from StemCell Technologies Inc. and Miltenyi BioTech GmbH. Underivatized antibodies are available from other sources such as Pharmagen, Inc. The resulting mixture is then centrifuged using the harvesting float device to harvest the enriched target component. Prior to centrifuging, the sample can be combined with a density gradient media as known in the art to enhance the separation of the target component.

[0038] The positive selection harvesting method in one embodiment of the invention utilizes a particulate carrier having a coating of antibody with an affinity toward the target component. In preferred embodiments, the particulate carrier is an amount of plastic microbeads with a coating of an antibody with a binding affinity for the rare cells to be harvested, and particularly tumor cells.

[0039] The density of the microbeads and the density of the float are coordinated to collect the microbeads in the axial bore of the float. The positive selection harvesting uses microbeads having a particle size of about 0.05 microns to about 7 microns, and typically about 4 to 5 microns, and a density less than white blood cells. The microbeads can have a density in the range of about 1.00 to about 1.05, and preferably about 1.02 to about 1.03. The microbeads and the captured rare cells have a density so that they are focused in the float when the sample and float are centrifuged. The float has an appropriate density so that the float settles in the sample

after centrifuging where the rare cells normally settle. In this embodiment, the microbeads are sufficiently light to float above the centrifuged layers of white and red blood cells. The float preferably has a density to float above the white and red blood cell layers to harvest the microbeads.

[0040] The microbeads are made of a suitable material that is non-reactive with the target component and particularly non-reactive with the rare cells. Suitable materials include polyacrylamides, polyurethanes, polysulfones, fluorinated or chlorinated resins, such as polyvinylchloride, polyethylene, polypropylene, polycarbonates and polyesters. The particle is typically about 4 to 5 microns, although the particle size can vary depending on the target component and internal diameter of the float. A number of commercially available microbeads have an antigen bonded to the surface of the microbeads. The antibody can be bonded directly to the surface of the microbead or through an intermediate coupling agent. Suitable antibody coated microbeads are commercially available from Miltenyi Biotec GmbH. An example of a suitable microbead is available from Miltenyi Biotec under the tradename MACS CD 27.

[0041] The antibody in the positive selection method has a binding affinity for the rare cells and is selected according to the target rare cells to be harvested from the sample. Examples of rare cells to be harvested include tumor cells, fetal cells and the like. Examples of tumor cells that can be bound to the particulate carrier can be of epithelial origin and can be localized or non-localized. The tumor cells can be of the bladder, brain, breast, colon, kidney, liver, lung, ovary, pancreas, prostate, rectum and stomach. Tumor cells can also be in the form of sarcoma, such as fibrosarcoma or rhabdosarcoma, hematopoietic tumor of the lymphoid or myeloid lineage, melanoma, teratocarcinoma, neuroblastoma, or glioma.

[0042] The microbeads preferably have a surface area sufficient to contain an amount of the selected antibody to bind an effective amount of the rare cells being targeted. The amount of the microbeads combined with the sample can vary with the affinity of the antibody, concentration of the rare cells in the sample, the nature of the sample, and the volume of the sample.

[0043] The method of the invention is suitable for use in harvesting rare cells from various bodily fluids, and particularly anticoagulated blood. Other fluids that can be analyzed for rare cell content include urine, saliva, lymph fluid, spinal fluid, semen, amniotic fluid, cavity fluids and tissue extracts.

[0044] The method is carried out using the centrifuge tube 12 and float 28. In preferred embodiments, tube 12 is evacuated or filled with an inert gas at a subatmospheric internal pressure. The sample to be tested is transferred from a primary collecting tube by a transferring device having a double piercing needle or cannula. The needle extends from the transferring device to the tube by piercing the stopper in tube 12. The low pressure

in tube 12 draws the fluid sample into tube 12. A thixotropic gel can be provided in tube 12 as known in the art to preserve band formation in the sample when centrifuged. Various other separation agents, dyes and the like can be added to tube 12 to promote separation and identification of components. The microbeads containing the antibody are provided in the tube 12 and are mixed with the fluid sample by gentle shaking or stirring. The sample is then incubated to bind the target component to the microbeads.

[0045] The tube, float and sample are centrifuged at a sufficient speed and for a length of time necessary to separate the constituents of the sample into layers and force the microbeads and the trapped target component into the axial bore of the float. The sample can be centrifuged at a speed to provide sufficient centrifugation force to cause separation of the layers. The tube is slowly stopped and removed from the centrifuge. A needle or cannula then pierces the stopper and is inserted into the axial bore to remove the sample containing the microbeads. The harvested sample is further processed and analyzed by various processes as known in the art. In one embodiment, the harvested cells are analyzed using a flow cytometer. The rare cells or other target components can be washed and separated from the microbeads and the binding antibody by known methods. The resulting harvested rare cells are significantly enriched compared to many prior processes and have a substantially lower contaminant level of red and white blood cells.

[0046] In a second embodiment of the invention, the method is a negative selection method for the enrichment of rare cells. The rare cells are enriched using a binding agent that is able to bind with the contaminating non-rare cells, such as red blood cells or white blood cells.

[0047] In preferred embodiments, the binding agent is able to bind to one or more white blood cell and/or red blood cell or that bind to surface antigens on the cells. The binding agents can be antibodies that are able to agglutinate the white blood cells or bind the white blood cells to red blood cells. The resulting larger and denser particles can be separated from the non-rare cells during centrifugation. Suitable antibodies that are able to bind with and capture the non-rare cells include antihuman antibodies. Examples of suitable antibodies that can be used to bind with white blood cells (leukocytes) include the leukocyte CD antibodies such as CD2, CD3, CD4, CD5, CD7, CD8, CD11a, CD11b, CD11c, CD14, CD15, CD16, CD19, CD20, CD28, CD36, CD42a, CD43, CD44, CD45, CD45R, CD45RA, CD45RB, CD45RO, CD57 and CD61. Other binding agents that can be used include a mixture of antihuman CD45, antihuman CD19, antihuman CD14 and antihuman CD3.

[0048] Preferably, the antibodies are bound to the surface of the microbeads as in the previous embodiments. The microbeads in the negative selection process have a particle size suitable for the sample and the target

component. Generally, the particle size ranges from about 0.05 microns to about 7 microns, and preferably about 4 microns to about 5 microns. In this embodiment, the beads preferably have a density greater than the density of white blood cells, and more preferably of about 1.07 to about 1.09 g/ml, and typically in the range of about 1.08 to about 1.09 g/ml. In this manner, the microbeads sink during centrifugation and the rare cells settle above the red and white blood cell layers. The float has density to float on the non-rare cells layers so that the rare cells settle in the axial bore of the float where they can be removed.

[0049] The method of the negative selection harvesting is similar to the positive selection discussed above. The sample fluid is provided in the tube and mixed with the microbeads containing the non-rare cell antibodies. After incubating, the tube containing the mixture is incubated and centrifuged for sufficient time to cause the layers to separate and the rare cells to collect in the axial bore of the float where the rare cells can be recovered.

[0050] In further embodiments of the invention, the method employs two microbeads having different affinity binding agents for capturing two different components. In one embodiment, an amount of first microbeads having an affinity binding agent with an affinity for rare cells, such as tumor cells, are mixed with the sample. The first microbeads have a density to separate from the white and red blood cells. The first microbeads have a particle size, density and affinity binding agent substantially the same as the microbeads of the positive selection of the previous embodiment. An amount of second microbeads having an affinity binding agent with an affinity for white blood cells is also mixed with the sample. The second microbeads have a density to separate the white blood cells, red blood cells, or a combination thereof from the rare cells. The resulting mixture is centrifuged with the float so that the first microbeads with the captured rare cells settle in the axial passage where they can be recovered. The second microbeads have a particle size, density and affinity binding agent substantially the same as the negative selection method of the previous embodiment. In this manner, the second microbeads separate from the first microbeads during centrifugation to separate the contaminating cells, such as the red and/or white blood cells from the target cells and the first microbeads. The first microbeads have a particle size and density to be collected in the axial passage of the float for recovering the target cells.

[0051] Referring to Figure 3, another embodiment of the centrifuge device 30 is shown. Device 30 is particularly suitable for various cell manipulations after separation from a sample. For example, rare cells can be separated from a sample and subjected to various detection and assay processes with device 30. In this embodiment, device 30 includes a hollow container 32 having a substantially rectangular shape. Container 32 includes a front wall 34, and an opposite rear wall 36 having a longitudinal length and a width. Opposite side walls

38 and a bottom wall 40 extend between front wall 34 and rear wall 36 to form an open cavity 42. Container 32 includes an open end 44 to receive a stopper 46 for closing cavity 42. Preferably, container 32 is made of a transparent material such as glass or plastic.

[0052] Container 32 is dimensioned to receive a volume of a biological sample suitable for analysis of a target component. Container 32 generally has a volume of about 8 ml to about 10 ml, and preferably about 9 ml. In the illustrated embodiment, side walls 38 of container 32 have a dimension to define a thickness of cavity 42 that is sufficiently thin to visualize, detect and analyze a target component through front wall 34. Examples of suitable detection and analysis methods include microscopy to visualize cells in the sample. Container 32 is typically about 7 cm to about 8 cm in length, and about 3 cm to about 4 cm in width. Side walls 38 are dimensioned so that cavity 42 has a thickness of about 3 mm to about 6 mm, and preferably about 4 mm.

[0053] Container 32 includes a movable float 48 that is able to slide within container 32 in the longitudinal dimension in a manner similar to the previous embodiment. Float 48 is dimensioned to fit within cavity 42 of container 32 and has an outer dimension corresponding to the inner dimension of container 32. As shown in Figures 3-5, float 48 has a base 50 with a substantially flat bottom surface 54. Base 50 includes an inclined leading end 56 and an inclined trailing end 58. A plurality of ribs 60 are coupled to top surface 54 of base 50.

[0054] As shown in Figure 5, ribs 60 extend in a longitudinal direction with respect to the longitudinal dimension of base 50. Ribs 60 are aligned in pairs to form channels 62 extending the length of base 50 between adjacent ribs. Ribs 60 have a height to fit closely against the inner surface of container 32. Channels 62 are dimensioned to separate and elongate the layers during centrifugation.

[0055] In this embodiment, a biological sample, such as a blood sample, is placed in container 32. An amount of microbeads 64 having an affinity binding agent for a target component is mixed with the sample. Container 32 is then centrifuged as in the previous embodiment to collect the microbeads 64 with the captured target component in longitudinal channels 62 of float 48. Microbeads 52 and the captured target component can then be analyzed by visualizing the target component within container 32 by microscopy methods as known in the art.

[0056] In the embodiments shown in Figure 5, the blood sample after centrifuging separates into a layer of red blood cells 66, a granulocyte cell fraction layer 68, a mononuclear cell fraction 70, a plasma fraction 72 and a platelet/plasma interface 74. In a positive selection process, microbeads 64 have an affinity for the target compound and collect in channels 62. Alternatively, microbeads 64 can have an affinity for white and/or red blood cells in a negative selection process. Channels 62 are formed between ribs 60 and are enclosed by top

wall 34 of container 32. Inclined leading edge 56 and inclined trailing edge 58 divert the sample through channels 62 as float 48 slides through container 32. Microbeads 64 are retained in a thin layer in channels 62 close to top wall 34 of container 32 so that the microbeads 64 can be visualized through top wall 34 by microscopy or other analytical methods as known in the art. Preferably, front wall 34 of container 32 is substantially flat to prevent the optical distortion normally associated with cylindrical containers.

Example

[0057] This example compares the harvested tumor cells from a sample with and without a negative selection. The harvester separations were compared for 6.0 mls of freshly collected whole blood that were spiked with 0, 50 and 500 cultured prostate tumor PC-3 cells. An antibody cocktail obtained from StemCell Technologies, Inc. under the tradename RosetteSep was mixed with each of the blood samples and incubated for 20 minutes at room temperature. A control blood sample was prepared without the antibody treatment. The antibody cocktail provided a negative selection to remove the unwanted white blood cells.

[0058] The blood samples were layered on top of a density media in a 16 x 100 PET Vacutainer tube obtained from Becton Dickinson containing a harvester float and 2 mls of POLYMORPHPREP™ density media. The samples were centrifuged in a swinging bucket centrifuge for 30 minutes at 20°C at a rate of about 650 g. The control sample showed the presence of white blood cells with the tumor cells in the harvester float. The antibody treatment demonstrated tumor cells collected in the harvester float with a greatly reduced white cell population. The tumor cells were removed from the harvester float. Flow cytometry demonstrated recovery of about 90% of the tumor cells.

[0059] While various embodiments have been chosen to illustrate the invention, it will be appreciated by those skilled in the art that various modifications and additions can be made without departing from the scope of the invention as defined in the appended claims.

Claims

1. A method of harvesting components from a sample material, said method comprising the steps of:

providing a sample material in a sampling container, said sampling container having a focusing device with a passage for receiving and elongating layers of sample components to be harvested from said sample,
providing at least one antibody in said sampling container, and mixing said antibody with said sample, wherein said antibody has an affinity

- for binding with at least one substance in said sample, and
centrifuging said container and sample at sufficient G forces to separate components of said sample and to force a target component from said sample into said passage.
2. The method of claim 1, further comprising the step of removing said target component from said passage.
3. The method of claim 1, wherein said focusing device is a float received in said container and having a dimension and shape complementing an interior of said container, said float including ribs defining a longitudinal channel between said float and said container for receiving and elongating said target component during centrifuging; and said method further comprising a particulate carrier, wherein said antibody is bound to said particulate carrier and has an affinity for capturing said target component, said method comprising centrifuging said container for sufficient time and at a sufficient speed to collect said particulate carrier and captured substance in said longitudinal channel.
4. The method of claim 1, wherein said container is a tube having an inner surface, and said focusing device is a float having an outer surface complementing said inner surface of said tube and having an axial through passage.
5. The method of claim 4, further comprising providing a particulate carrier and mixing said particulate carrier with said sample, wherein said at least one antibody is bound to a surface of said particulate carrier.
6. The method of claim 5, wherein said carrier comprises an effective amount of microbeads having a density greater than a density of white blood cells and wherein said antibody has an affinity for white blood cells.
7. A method of harvesting a target component from a sample, said method comprising the steps of:
- providing a sample in a sampling tube, said sampling tube containing a float dimensioned to fit within said sampling tube and having a through passage for receiving and elongating layers of blood constituents to be harvested from said sample,
mixing said sample with at least one particulate carrier containing an antibody having a binding affinity for a specific sample constituent,
centrifuging said tube and sample at sufficient G forces to move said float toward one end of
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- said tube and to force a target component from said sample into said through passage, and removing said target component from said through passage.
8. The method of claim 7, further comprising the step of incubating said sample prior to centrifuging.
9. The method of claim 7, wherein said antibody has an affinity for said target component, and further comprising the step of separating said target constituent from said particulate carrier.
10. A method of harvesting a target component from a whole blood sample, said method comprising the steps of:
- providing a whole blood sample in a sampling tube, said sampling tube containing a float dimensioned to fit within said sampling tube and having a through passage for receiving and elongating layers of blood constituents to be harvested from said sample,
mixing said sample with an amount of first carrier beads having a coating of a first antibody that has a binding affinity for a target constituent in said sample, and an amount of second carrier beads having a coating of said second antibody that has a binding affinity for white blood cells,
centrifuging said tube and sample at sufficient G forces to move said float toward one end of said tube and to force said first carrier beads and target constituent into said through passage, and
removing said first carrier beads and target constituent from said through passage.

FIG. 1

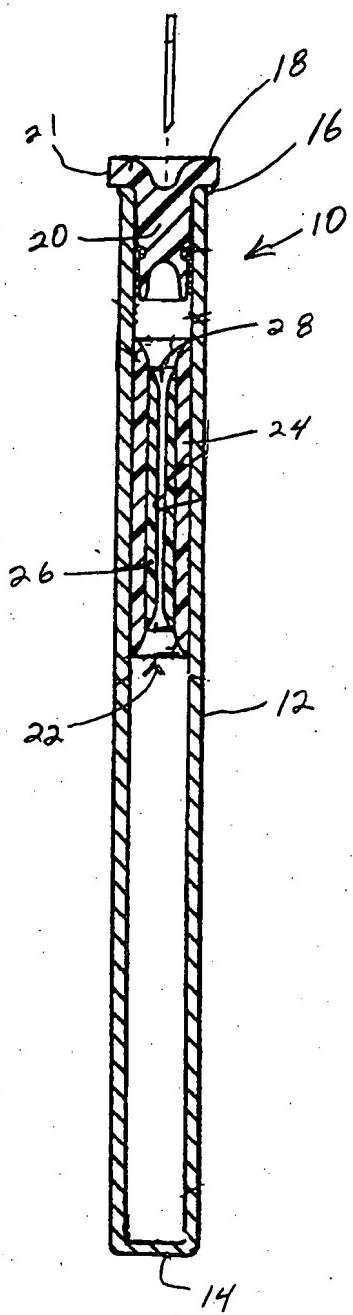


FIG. 2

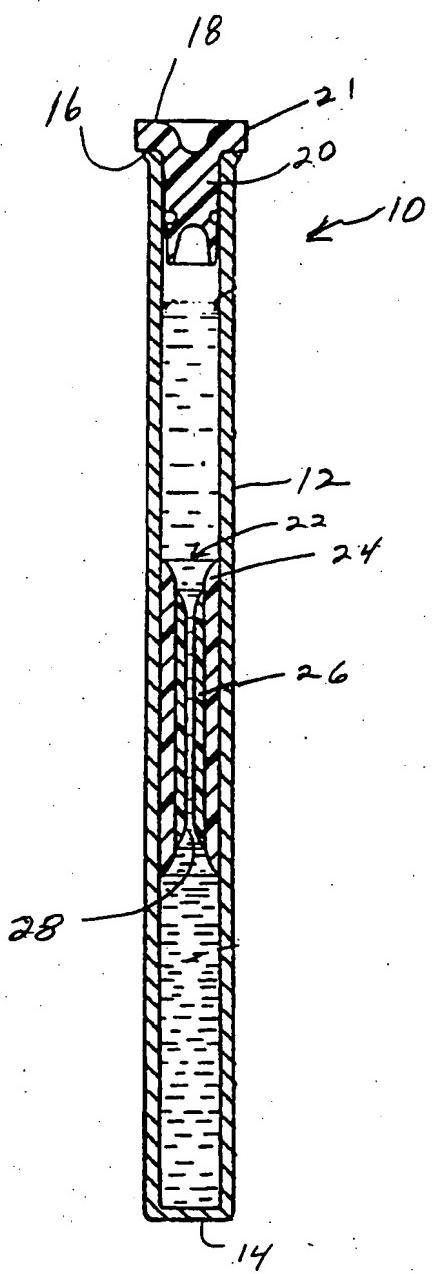
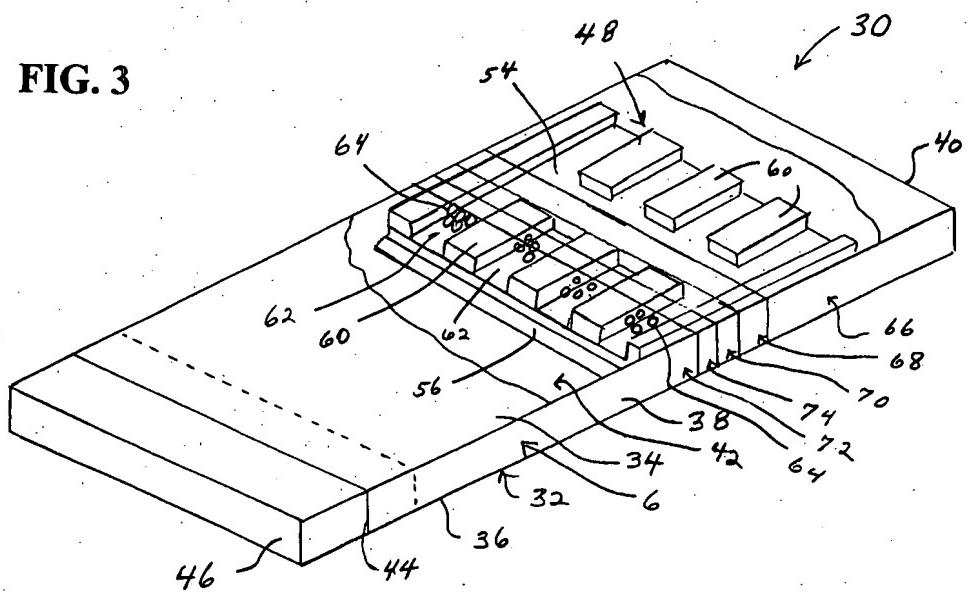
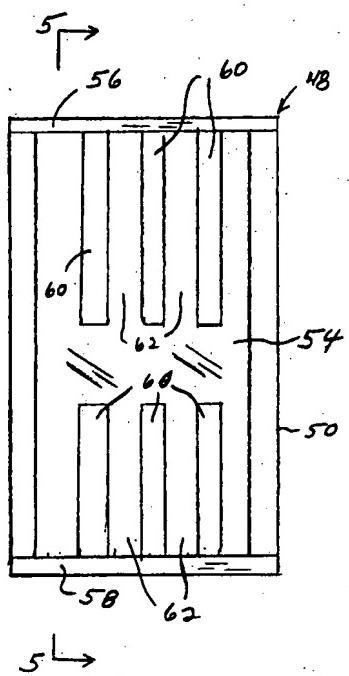
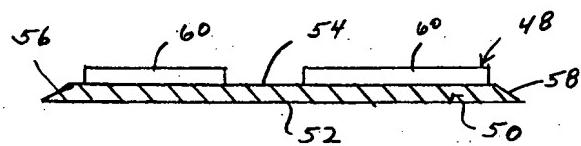


FIG. 3**FIG. 4****FIG. 5**

Method for the quantitative detection of vital epithelial tumour cells in a body fluid

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Abstract of EP1262776

Quantitative determination (M1) of live epithelial tumor cells (A) in a body fluid by laser-scanning cytometry, is new. (M1) comprises labeling (A) in a predetermined amount of body fluid by adding: (i) anti-human epithelial antibody (Ab) coupled to magnetic beads; and (ii) same antibody coupled to a fluorophore. (A) are then concentrated magnetically, the resulting suspension immobilized on a carrier by spontaneous adhesion and (A) determined by laser-scanning cytometry. The number of (A) relative to the amount of body fluid sample is calculated.

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(54) Verfahren zum quantitativen Nachweis vitaler epithelialer Tumorzellen in einer Körperflüssigkeit

(57) Die Erfindung betrifft ein Verfahren zum Nachweis epithelialer Tumorzellen in einer Körperflüssigkeit mit folgenden Schritten:

- a) Bereitstellen einer bestimmten Menge einer Körperflüssigkeit,
- b) Markieren der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an magnetische Partikel gebunden sind, zu der Körperflüssigkeit,
- c) Markieren der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an ein Fluorochrom gebunden sind, zu der Körperflüssigkeit,
- d) Magnetische Anreicherung der vitalen epithelialen Tumorzellen,

e) Immobilisierung der so erhaltenen Suspension auf einem Trägermaterial durch selbständiges Anhaften der vitalen Tumorzellen,

f) Erfassung der vitalen epithelialen Tumorzellen mittels Laser-Scanning-Cytometrie und Berechnung der Anzahl dieser Zellen in bezug auf die Menge der bereitgestellten Körperflüssigkeit.

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Beschreibung

[0001] Die Erfindung betrifft ein Verfahren zum quantitativen Nachweis vitaler, epithelialer Tumorzellen in einer Körperflüssigkeit.

[0002] Die Erfindung betrifft allgemein das Gebiet der Indikation solider Tumoren. Bekanntlich ist die Metastasierung bei soliden Tumoren der Hauptgrund für hohe Krebssterblichkeit. Sie wird durch Zellen verursacht, die in den Lymphknoten disseminiert werden und/oder im peripheren Blut zirkulieren. Von den zirkulierenden Tumorzellen kann unter Umständen ein Teil in entfernte Kompartimente gelangen, wo sie erneut zu wachsen beginnen. Derartige Kompartimente sind bei einigen Tumoren bekannt. Bei Brustkrebs und Dickdarmkrebs ist ein solches Kompartiment das Knochenmark. Die Häufigkeit der Tumorzellen in bezug auf normale Knochenmarkszellen beträgt höchstens 10^{-3} bis 10^{-7} Tumorzellen/normale Knochenmarkszellen. Um Proben für eine Knochenmarksdiagnostik zu erhalten, ist ein spezieller Eingriff in Verbindung mit oder nach einer Operation erforderlich. Eine regelmäßige Kontrolle würde eine Wiederholung eines derartigen Eingriffs erfordern. Wegen der damit verbundenen Unannehmlichkeiten für den Patienten sowie des Kosten- und Zeitaufwands ist man bestrebt, die Anzahl operativer Eingriffe möglichst gering zu halten.

[0003] Eine weitere Möglichkeit bietet eine Untersuchung des wesentlich leichter zugänglichen peripheren Blutes. Dabei tritt allerdings das Problem auf, daß darin nachweisbare Tumorzellen nur in äußerst geringer Anzahl vorhanden sind. Erschwerend kommt hinzu, daß die im peripheren Blut zirkulierenden Tumorzellen das Transplantat bei einer Hochdosis-Chemotherapie oder einer autologen peripheren Blutstammzelltransplantation kontaminieren können. Es sind deshalb hochempfindliche Systeme notwendig, um eine derart geringe Anzahl an restlichen Tumorzellen nachzuweisen.

[0004] Das derzeit empfindlichste Nachweisverfahren ist die Polymerase-Kettenreaktion (PCR). Bei hämatologischen Malignomen zeigt die PCR von Gensequenzen, die mit dem Tumor assoziiert sind, eine hohe Empfindlichkeit beim Nachweis einer geringen Zahl an Tumorzellen. Allerdings ist bei soliden Tumoren die PCR mit Problemen hinsichtlich der Anwendbarkeit, der Spezifität und des klinischen Einflusses verbunden. Als weiteres Verfahren kann auch die gewebe- und reifungsabhängige Expression der Oberflächen- oder intrazellulären Antigene genutzt werden, um aberante Zellen vom normalem Gewebe immunologisch zu unterscheiden. Die Häufigkeit von 10^{-3} bis 10^{-8} , mit der Tumorzellen aus soliden Tumoren normalerweise in peripherem Blut zu erwarten sind, erfordert aber die Prüfung einer hohen Anzahl negativer Zellen, um eine positive Zelle bzw. eine Tumorzelle zu finden. Ein derartiger immunologischer Nachweis der Tumorzellen muß mit Hilfe eines Mikroskops vorgenommen werden. Er ist mit einem sehr hohen Aufwand verbunden. Dabei kön-

nen Tumorzellen aufgrund ihrer geringen Anzahl übersehen werden. Die Genauigkeit eines solchen Nachweisverfahrens vergleichsweise gering ist. Auch die Verwendung von Bildanalyseverfahren verbessert die Sensitivität und die Geschwindigkeit nur unwesentlich.

[0005] Bei bekannten Verfahren wird die Anzahl der Tumorzellen in bezug auf die Anzahl aller Zellen, beispielsweise der Leukozyten, ermittelt. Die Anzahl der Leukozyten kann aber zum Beispiel bei einer Hochdosis-Chemotherapie stark schwanken, so daß die Anzahl der Tumorzellen in bezug auf die Anzahl von Leukozyten nur bedingt aussagefähig ist.

[0006] Aufgabe der Erfindung ist es, die Nachteile nach dem Stand der Technik zu beseitigen. Es soll insbesondere ein verbessertes Verfahren zum quantitativen Nachweis vitaler epithelialer Tumorzellen in einer Körperflüssigkeit angegeben werden, dessen Genauigkeit und Geschwindigkeit die bisher bekannter Verfahren übertrifft.

[0007] Diese Aufgabe wird durch die Merkmale des Anspruchs 1 gelöst. Zweckmäßige Ausgestaltungen der Erfindung ergeben sich aus den Merkmalen der Ansprüche 2 bis 11.

[0008] Nach Maßgabe der Erfindung ist ein Verfahren zum quantitativen Nachweis vitaler epithelialer Tumorzellen in einer Körperflüssigkeit mit folgenden Schritten vorgesehen:

a) Bereitstellen einer bestimmten Menge einer Körperflüssigkeit,

b) Markieren der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an magnetische Partikel gebunden sind, zu der Körperflüssigkeit,

c) Markieren der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an ein Fluorochrom gebunden sind, zu der Körperflüssigkeit,

d) Magnetische Anreicherung der vitalen epithelialen Tumorzellen,

e) Immobilisieren der so erhaltenen Suspension auf einem Trägermaterial durch selbständiges Anhaften der vitalen Tumorzellen,

f) Erfassen der vitalen epithelialen Tumorzellen mittels Laser-Scanning-Cytometrie und Berechnen der Anzahl dieser Zellen in bezug auf die Menge der bereitgestellten Körperflüssigkeit.

[0009] Das vorgeschlagene Verfahren bietet die Möglichkeit, die Anzahl von vitalen epithelialen Tumorzellen direkt in einer Körperflüssigkeit, wie beispielsweise Blut, Knochenmark, Knochenmark-Aspirat, Transsudat, Exsudat, Lymphe, Apheresat, Aszites, Urin, Speichel und

Drainageflüssigkeiten aus Wundsekreten, zu bestimmen. Die eingesetzten Antikörper binden spezifisch an vitale tumorverdächtige Zellen. Vitale Tumorzellen können damit von toten Tumorzellen getrennt werden. Die Anzahl der vitalen Tumorzellen kann in bezug auf das Volumen der eingesetzten Körperflüssigkeit angegeben werden. Das erfindungsgemäße Verfahren liefert standardisierte Werte. Überdies kann das Trägermaterial, auf dem die untersuchte Körperflüssigkeit nach Markierung, Anreicherung und Trennung aufgetragen wird, zu Dokumentationszwecken aufbewahrt werden, so daß es für eine spätere Begutachtung und weitere Charakterisierung zur Verfügung steht. Bei den bisherigen Verfahren kann demgegenüber zumeist nur ein Meßprotokoll aufbewahrt werden.

[0010] Mit dem Verfahren der vorliegenden Erfindung können sehr geringe Mengen an Tumorzellen in einer Körperflüssigkeit nachgewiesen werden. Zu Testzwecken wurden beispielsweise zehn Tumorzellen zu 20 ml Vollblut gegeben. Diese zehn Tumorzellen konnten mit dem erfindungsgemäßen Verfahren vollständig nachgewiesen werden. Dieses Ergebnis ist mit dem vergleichbar, das mittels PCR erreicht werden kann, wobei jedoch die mit diesem Verfahren verbundenen Nachteile vermieden werden können. Das erfindungsgemäße Verfahren erlaubt die quantitative Bestimmung vitaler epithelialer Tumorzellen in einer Körperflüssigkeit.

[0011] Zweckmäßigerweise wird die Körperflüssigkeit, insbesondere peripheres Blut, vor der Markierung der vitalen epithelialen Tumorzellen mit Antikörpern lyisiert, um beispielsweise Erythrozyten abzutrennen. Die erhaltene Suspension wird anschließend zentrifugiert, der Überstand abgetrennt und verworfen. Es ist nicht erforderlich, daß alle Erythrozyten entfernt werden, da diese das Verfahren nicht beeinflussen.

[0012] In einer Variante der Erfindung werden die Tumorzellen vor der magnetischen Anreicherung sowohl mit antihumanen epithelialen Antikörpern, die an magnetische Partikel gebunden sind ("magnetic beads" oder "Microbeads"), als auch mit antihumanen epithelialen Antikörpern, die an ein Fluorochrom gebunden sind, markiert. Die magnetischen Partikel haben vorzugsweise einen Durchmesser, der kleiner als 70 nm ist. Die Tumorzellen tragen über die Antigen-Antikörper-Bindung sowohl magnetische Partikel als auch einen Fluoreszenzfarbstoff. Die Tumorzellen werden anschließend durch magnetische Zellseparation angereichert, indem sie z.B. auf eine Säule gegeben werden, die sich in einem starken, z.B. durch einen Permanentmagnet gebildeten, Magnetfeld befindet. Die Zellen der Körperflüssigkeit, an die keine magnetischen Partikel gebunden sind, werden aus der Säule herausgespült. Die markierten Zellen verbleiben wegen der Wirkung des Magnetfelds in der Säule. Nach dem Entfernen des Magnetfelds können die in der Säule verbliebenen Tumorzellen ausgespült werden.

[0013] Alternativ kann die magnetische Zellseparation erfolgen, bevor die vitalen epithelialen Tumorzellen

durch Zugabe antihumaner epithelialer Antikörper, die an ein Fluorochrom gebunden sind, markiert werden. Dazu ist es ausreichend, wenn die Tumorzellen mit den magnetischen Partikeln markiert werden.

- 5 [0014] Zweckmäßigerweise wird vor dem Markieren der vitalen epithelialen Tumorzellen ein FcR-Blockierungsreagenz zu der Körperflüssigkeit zugesetzt.
- 10 [0015] Als Antikörper werden vorzugsweise antihumane epitheliale Antikörper (HEA) verwendet, die von der Maus stammen. Als Fluorochrom wird vorzugsweise Fluoresceinisothiocyanat (FITC) eingesetzt.
- 15 [0016] Nach der Anreicherung und Einfärbung der vitalen epithelialen Tumorzellen wird die Zellsuspension auf einen Träger aufgebracht. Dabei handelt es sich zweckmäßigerweise um einen Objekträger aus Glas, der vorzugsweise mit Poly-L-Lysin beschichtet ist.
- 20 [0017] Die Anzahl der Tumorzellen auf dem Träger bzw. Trägermaterial wird mittels Laser-Scanning-Cytometrie bestimmt. Zur Konturierung der auf dem Trägermaterial befindlichen Zellen wird zweckmäßigerweise das Vorwärtsstreulicht (forward scatter) als Schwellwertparameter bei einer geringen Vergrößerung genutzt. Die Hintergrundfluoreszenz kann dynamisch bestimmt werden, um die maximale Fluoreszenzintensität und/oder die Gesamtfluoreszenz durch Integration über jede Zelle zu bestimmen. Das ermöglicht es, Veränderungen der Hintergrundfluoreszenz zu korrigieren, so daß die Fluoreszenz für jede Zelle unter den gleichen Bedingungen berechnet werden kann und äquivalente Ergebnisse für jede Zelle erhalten werden. Die grüne Fluoreszenz der FITC-HEA-markierten Zellen wird vorzugsweise unter Verwendung eines 530/30 nm-Bandpaßfilters erfaßt.
- 25 [0018] Die angereicherten und FITC-markierten Zellen (FITC-positive Zellen) können außerdem mit einem weiteren Fluoreszenzfarbstoff angefärbt werden. Beispielsweise kann die DNA der Zellkerne mit einem DNA-spezifischen Farbstoff wie Propidiumiodid angefärbt werden. Die rote Fluoreszenz der so angefärbten Zellen wird ebenfalls mittels Laser-Scanning-Cytometrie unter Verwendung eines 625/28 nm-Bandpaßfilters erfaßt. Als Schwellwertparameter zur Konturierung dient das Vorwärtsstreulicht. Die gemessene rote und grüne Fluoreszenz werden miteinander verglichen und die Anzahl der positiven Ereignisse ermittelt.
- 30 [0019] Die Anzahl an markierten positiven Zellen ist im Vergleich zu nach dem im Stand der Technik bekannten Verfahren aufgrund der Anreicherung pro Volumen besonders hoch, so daß die Geschwindigkeit und die Genauigkeit des erfindungsgemäßen Verfahrens deutlich höher sind. Die Anzahl der gefundenen markierten Zellen wird dann auf das ursprünglich eingesetzte Volumen an Körperflüssigkeit zurückgerechnet. Das erlaubt es zu verschiedenen Zeitpunkten ermittelte Nachweisergebnisse zu vergleichen. Es kann rasch der Erfolg z.B. einer Tumortherapie erkannt werden.
- 35 [0020] Die Morphologie der erfaßten positiven Zellen kann anschließend durch hämatologische Färbeverfah-

ren wie die May-Grünwald-Färbung bestimmt werden.

[0021] Das erfindungsgemäße Verfahren erlaubt vor teilhafterweise die Durchführung mehrerer quantitativer Nachweise hintereinander. Die Zellen können quantitativ im Hinblick auf verschiedene Parameter untersucht werden. Dazu können die Zellen nach einer ersten quantitativen Auswertung z.B. mit einer einen zweiten Nachweisstoff, z.B. einen Fluoreszenzfarbstoff, enthal tenden Lösung beaufschlagt werden, welcher z.B. spezifisch für Malignität ist. Die Koordinaten der Zellen sind bereits von der Durchführung des ersten quantitativen Nachweises bekannt und im Computer gespeichert. Es kann jede Zelle sofort aufgefunden und deren Reaktion gegenüber weiteren Nachweissubstanzen erfaßt und quantitativ ausgewertet werden. Als weitere Nachweis verfahren können insbesondere FISH oder TUNEL durchgeführt und quantitativ ausgewertet werden.

[0022] Nachfolgend wird die Erfindung anhand von Ausführungsbeispielen näher erläutert.

Beispiel 1

[0023] In diesem Beispiel wird ein Verfahren zum Nachweis von epithelialen Tumorzellen in Blut beschrieben, bei dem die magnetische Anreicherung der vitalen epithelialen Tumorzellen nach der Markierung der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, an die ein Fluorochrom gebunden ist, erfolgt.

a. Herstellen einer Verdünnungsreihe von Tumorzellen enthaltendem Vollblut

[0024] Peripheres Blut wurde mit verschiedenen Anteilen an Tumorzellen gemischt. Die Tumorzellen stammten aus einer Brustkrebszelllinie (SK Br2-artig). Die Leukozyten des peripheren Blutes und die Tumor zellen wurden gezählt. In einem ersten Verdünnungsschritt wurden 6×10^5 Tumorzellen mit 100 µl Vollblut, das 6×10^5 Leukozyten enthielt, gemischt. Im nächsten Verdünnungsschritt wurden jeweils 6×10^4 Tumorzellen und 6×10^3 Tumorzellen zu 100 µl Blut gegeben. Im anschließenden Verdünnungsschritt wurden 6×10^3 und 6×10^2 Tumorzellen zu 1 ml Blut sowie 6×10^2 und 60 Tumorzellen zu 10 ml oder 20 ml Blut gegeben, wobei im letzten Fall eine Verdünnung von 5 Tumorzellen auf 10^7 normale Zellen erhalten wurde.

b. Magnetische Anreicherung und Anfärbung

[0025] Zur Trennung an einer magnetischen Säule wurden 400 µl mit bis zu 5×10^7 Zellen aus der in lit. a. erhaltenen Verdünnungsreihe mit 100 µl Blockierungs reagens 30 min in der Kälte inkubiert. Die Zellen wurden dann mit magnetischen Partikeln, an die antihumane epithelialen Antikörper gebunden sind, (100 µl HEA-Microbeads, Miltenyi) behandelt, 15 min inkubiert und 10mal mit dem Markierungspuffer gewaschen. Die

Säulen, an denen ein Magnet angebracht ist, wurden gewaschen und die magnetischen Partikel in den Säulen gesammelt. Die negativen Zellen wurden dann durch Spülen mit 5mal 500 µl Puffer eluiert und die Säulen vom Magnet getrennt. Die in den Säulen verbliebenen Zellen wurden mit zusätzlichem Puffer herausge spült und mit 50 µl FITC-konjugierten antihumanen epithelialen Mäuseantikörpern (HEA-FITC-Antikörper, Miltenyi) angefärbt.

c. Immobilisierung der Zellen und Bestimmung der Anzahl der Zellen

[0026] Die Zellen wurden auf Objekträger aufgetragen. Nach dem Auftragen von 100 µl der Zellsuspension hafteten die vitalen Zellen nach 10 bis 15 min auf der Oberfläche des Objekträgers. Für optimale Messungen muß eine Einzelzellsuspension auf dem Objekträger aufgetragen werden, wobei die Zellen einen Abstand untereinander aufweisen, der etwa dem 2- bis 3fachen des Durchmessers einer Zelle entspricht.

[0027] Die anhaftenden Zellen wurden unter Verwendung eines Laser-Scanning-Cytometers (LSC Compucyte Corp.) gemessen. Die Konturierung der auf dem Objekträger haftenden Zellen wurde unter Verwendung des Vorwärtsstreuulichtes (forward scatter) als Schwellwertparameter bei einer zwanzigfachen Vergrößerung durchgeführt. Die Hintergrundfluoreszenz wurde dynamisch bestimmt, um sowohl die maximale Fluoreszenzintensität als auch die Gesamtfluoreszenz auf Basis einer Zelle zu berechnen. Dies ermöglicht es, die Schwankungen der Hintergrundfluoreszenz zu korrigieren, so daß die Berechnung der Fluoreszenz für alle Zellen gleichwertig erfolgt. Die grüne Fluoreszenz der positiven HEA-FITC-Zellen wurde mit einem 530/30 nm Bandpaßfilter erfaßt und mit einem Photomultiplier verstärkt.

[0028] Anschließend wurden die Zellen auf den Objekträgern zentrifugiert und in eine 1 mg/ml Propidiumiodid enthaltende PBS-Lösung getaucht. Der Schwellwert für die Konturierung der Zellen basierte ebenfalls auf dem Vorwärtsstreuulicht. Die rote Fluoreszenz wurde mit einem 625/30 nm Bandpaßfilter erfaßt und mit einem zweiten Photomultiplier verstärkt. Die rote und grüne Fluoreszenz wurden mittels eines Computerprogrammes (WinCyte, Compucyte Corporation) angeglichen und als Streulichtdiagramme, Histogramme, Prozentwerte und Mittelwerte der FITC-positiven und FITC-negativen Zellen dargestellt, wobei die Berechnung nur auf dem Bereich beruht, der Einzelzellen umfaßt.

d. Ergebnis

[0029] Die Korrelation zwischen der berechneten Anzahl von Tumorzellen in einer Probe der Verdünnungsreihe und der mittels dem Verfahren ermittelten Anzahl war sehr hoch (> 0,99). Sogar bei der 10^{-8} -fachen Ver-

dünnung konnten 50 von 60 Zellen nachgewiesen werden.

Beispiel 2

[0030] In diesem Beispiel wird ein Verfahren zum Nachweis von epithelialen Tumorzellen in Blut beschrieben, wobei die magnetische Anreicherung der vitalen epithelialen Tumorzellen vor der Markierung der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an ein Fluorochrom gebunden sind, erfolgt.

a. Markierung der Tumorzellen

[0031] 20 ml peripheres Blut wurde mit 40 ml Erylysuspuffer (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM Na₂-EDTA) versetzt und in der Kälte 7 min bei 2000 rpm zentrifugiert. Der Überstand wurde verworfen und das Sediment in PBS-EDTA-Lösung (50 ml PBS mit 200 µl 0,5 M EDTA) aufgenommen, so daß das Gesamtvolumen 900 µl betrug. Zu 300 µl dieses Gemisches wurden 100 µl FcR-Blockierungsreagenz (Milteny), 100 µl der magnetischen Partikel, an die antihumane epithelialen Antikörper gebunden sind, (100 µl HEA-Microbeads, Milteny) zugegeben und gemischt. Anschließend wurden zu dieser Suspension 50 µl FITC-konjugierte antihumane epithelialen Mäuseantikörper (HEA-FITC-Antikörper, Milteny) zugegeben und 15 min in der Kälte inkubiert.

b. Magnetische Anreicherung

[0032] Die Separationsäulen wurden in den Magneten (ctaMACS, Milteny) gestellt und zweimal mit 500 µl PBS-EDTA-Lösung gewaschen. Die in a. erhaltenen Suspension wurde mit 500 µl PBS-EDTA-Lösung aufgeschwemmt und auf die Säule gegeben. Die Säule wurde dreimal mit 500 µl PBS-EDTA-Lösung gewaschen und aus dem Magneten entnommen. Die Säule wurde anschließend mit 200 µl PBS-EDTA-Lösung gespült, um die markierten Zellen aufzunehmen.

c. Immobilisierung der Zellen und Bestimmung der Anzahl der Zellen

[0033] Auf einen mit Poly-L-Lysin beschichteten Objekträger (Labor Schubert) wurden 100 µl der bei lit. b. erhaltenen Lösung aufgetragen und gleichmäßig verteilt. Die Anzahl der Tumorzellen wurde wie in Beispiel 1, lit. c bestimmt und die Anzahl in bezug auf das eingesetzte Volumen an peripherem Blut berechnet.

Patentansprüche

1. Verfahren zum quantitativen Nachweis vitaler epithelialer Tumorzellen in einer Körperflüssigkeit mit

folgenden Schritten:

- a) Bereitstellen einer bestimmten Menge einer Körperflüssigkeit,
 - b) Markieren der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an magnetische Partikel gebunden sind, zu der Körperflüssigkeit,
 - c) Markieren der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an ein Fluorochrom gebunden sind, zu der Körperflüssigkeit,
 - d) Magnetische Anreicherung der vitalen epithelialen Tumorzellen,
 - e) Immobilisieren der so erhaltenen Suspension auf einem Trägermaterial durch selbständiges Anhaften der vitalen Tumorzellen,
 - f) Erfassen der vitalen epithelialen Tumorzellen mittels Laser-Scanning-Cytometrie und Berechnen der Anzahl dieser Zellen in bezug auf die Menge der bereitgestellten Körperflüssigkeit.
2. Verfahren nach Anspruch 1, wobei mittels Laser-Scanning-Cytometrie die maximale Fluoreszenzintensität und/oder die Gesamtfluoreszenz pro Zelle bestimmt wird.
 3. Verfahren nach einem der vorstehenden Ansprüche, wobei die Hintergrundfluoreszenz dynamisch bestimmt wird, so daß für jede Zelle äquivalente Fluoreszenzwerte erhalten werden.
 4. Verfahren nach einem der vorstehenden Ansprüche, wobei die magnetische Anreicherung der vitalen epithelialen Tumorzellen vor der Markierung der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an ein Fluorochrom gebunden sind, zu der Körperflüssigkeit erfolgt.
 5. Verfahren nach einem der vorstehenden Ansprüche, wobei die magnetische Anreicherung der vitalen epithelialen Tumorzellen nach der Markierung der vitalen epithelialen Tumorzellen durch Zugabe antihumaner epithelialer Antikörper, die an ein Fluorochrom gebunden sind, zu der Körperflüssigkeit erfolgt.
 6. Verfahren nach einem der vorstehenden Ansprüche, wobei die Körperflüssigkeit vor dem Markieren der vitalen epithelialen Tumorzellen mit Antikörpern lysiert, zentrifugiert und der Überstand abgetrennt

und verworfen wird.

7. Verfahren nach einem der vorstehenden Ansprüche, wobei zu der Körperflüssigkeit vor dem Markieren der vitalen epithelialen Tumorzellen ein Blockierungsreagenz zugesetzt wird. 5
8. Verfahren nach einem der vorstehenden Ansprüche, wobei zur Markierung der vitalen epithelialen Tumorzellen mit antihumanen epithelialen Antikörpern von der Maus stammende antihumane epithiale Antikörper verwendet werden. 10
9. Verfahren nach einem der vorstehenden Ansprüche, wobei als Fluorochrom Fluoresceinisothiocyanat (FITC) eingesetzt wird. 15
10. Verfahren nach einem der vorstehenden Ansprüche, wobei die Körperflüssigkeit aus der folgenden Gruppe ausgewählt wird: Blut, Knochenmark, Knochenmark-Aspirat, Transsudat; Exsudat, Lymphe, Apheresat, Aszites, Urin, Speichel und Drainageflüssigkeiten aus Wundsekreten. 20

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(54) SYSTEMS AND METHODS FOR CELL SUBPOPULATION ANALYSIS

SYSTEME UND VERFAHREN ZUR ZELLTEILBEVÖLKERUNGSANALYSE

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Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description**BACKGROUND OF THE INVENTION****5 1. Field of the Invention**

[0001] The present invention relates generally to fluidic processing and, more particularly, to methods and apparatuses concerning an integrated fluidic device capable of enriching and isolating a suspect cell subpopulation from a suspension of cells and quantitatively analyzing that subpopulation for marker proteins and mRNAs for the purpose of detection and diagnosis of conditions such as cancer.

10 2. Description of Related Art

[0002] The identification of increasing numbers of genes that influence disease states and the approach of the post-genomic era make evident the need for faster and automated technologies that will allow dissemination of the gains of molecular diagnosis. If sufficiently small, automatic and inexpensive devices can be developed for molecular screening, they would not only revolutionize the diagnosis and prognosis of cancer and other diseases but also would enable molecular methods to be disseminated completely - even to the point of care.

[0003] Although some devices such as gene chips and chip embodiments of the polymerase chain reaction (PCR) are beginning to enter use, many of the methods developed so far are labor intensive and are not readily suited to automated, continuous monitoring, or high throughput applications. Clearly, a wide range of enabling technologies is needed before integrated instruments capable of automated sample preparation and molecular analysis of clinical samples become a reality.

25 SUMMARY OF THE INVENTION

[0004] Technology that is the subject of the present addresses issues related to the creation of multiple-use diagnostic systems for combined sample preparation and detection of molecular markers. Disclosed herein are systems, methods, and devices capable of performing fully automated assays. These devices offer the advantages of small size, low sample volume requirements, and the potential for mass production at low cost. Such low-cost systems are applicable to reusable or disposable medical devices.

[0005] In one embodiment, such a system may include the following subsystems: (1) a prefilter stage to concentrate suspect cells; (2) a high discrimination separator stage to fractionate cell subpopulations; (3) a stage to burst cells and mobilize molecular components; and (4) a stage for automated analysis of protein and mRNA molecular diagnostic markers.

[0006] Important technologies for the development of such a system, and others made apparent by the present disclosure include the following: a prefiling methodology to trap suspected cancer cells from blood or dispersed lymph node cells; a force balance method that exploits dielectric properties of the suspect cells, and, if needed, their immunomagnetic labeling properties, to fractionate them into a microfluidic isolation and analysis chamber; and a dielectric indexing and manipulation method for carrier beads that, when combined with certain established molecular assay methods, allows for the parallel quantification of multiple molecular markers.

[0007] U.S. Patent No. 5,858,192 entitled "Method and Apparatus for Manipulation Using Spiral Electrodes" discloses the preamble of the independent claim 1.

[0008] As certain technology disclosed herein builds upon work involving dielectrophoretic trapping, dielectrophoretic field-flow fractionation (DEP-FFF), traveling wave methods, and other work performed by the inventors, United States Patent No. 5,993,630 entitled "Method and Apparatus for Fractionation Using Conventional Dielectrophoresis and Field Flow Fractionation"; U.S. Patent No. 5,888,370 entitled "Method and Apparatus for Fractionation Using Generalized Dielectrophoresis and Field Flow Fractionation"; U.S. Patent No. 5,993,632 entitled "Method and Apparatus for Fractionation Using Generalized Dielectrophoresis and Field Flow Fractionation"; United States Application No. 09/249,955 filed February 12, 1999 and entitled "Method and Apparatus for Programmable Fluidic Processing"; United States Application No. 09/395,890 filed September 14, 1999 and entitled "Method and Apparatus for Fractionation Using Generalized Dielectrophoresis-and Field Flow-Fractionation"; United States Provisional Application No. 60/211,757 filed June 14, 2000 and entitled "Method and Apparatus for Combined Magnetophoretic and Dielectrophoretic Manipulation of Analyte Mixtures"; United States Provisional Application No. 60/211,515 filed June 14, 2000 and entitled "Dielectrically-Engineered Microparticles"; United States Provisional Application No. 60/211,516 filed June 14, 2000 and entitled "Apparatus and Method for Fluid Injection."

[0009] Dielectric indexing represents a new approach to identifying individual molecular tests in a parallel molecular analysis scheme that substitutes dielectric indexing of carrier beads for the spatial indexing used on a gene chip. This

new approach allows different subpopulations of beads, each carrying a probe of a different molecular marker, to be identified and manipulated within the carrier medium using a dielectric fingerprint unique to each bead/probe type. The need to immobilize different molecular probes in a tightly specified pattern on a fixed substrate as demanded, for example, by gene chip technology, is thereby eliminated. Mixtures of probes, each probe carried on a separately indexed bead type, may be injected into and flushed from a reusable assay system in order to examine any desired panel of molecular markers.

[0010] The use of bead dielectric properties as an indexing parameter not only provides the capability of manipulating beads through dielectrophoresis or another suitable manipulation force, but also offers a new alternative to optical or fluorescent bead indexing methods that might interfere with low light emissions in fluorescent probe assays.

[0011] Technology disclosed herein builds upon and synthesizes aspects of many disciplines including field-flow fractionation (physical chemistry), dielectrophoresis and magnetophoresis (physics), microfluidics (mechanical and fluid engineering), microfabrication (photolithography, MEMS and magnetic materials science), control electronics (electrical engineering), antibody and nucleic acid binding and linking (immunology and molecular biology), cell biology (cell culture and cytology), flow cytometry, and oncology.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The following drawings form part of the present specification and are included by way of example and not limitation to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings, in which like references indicate similar elements, in combination with the detailed description of specific embodiments presented herein.

FIG. 1 is graph showing different DEP crossover frequencies. It compares the crossover frequencies for nine human tumor cell types and normal peripheral blood mononuclear cells.

FIGS. 2A-2D are pictures showing the removal of cultured breast cancer cells from blood by cDEP affinity trapping.

FIG. 3 is a schematic illustrating some operating principles of cDEP/FFF fractionation.

FIG. 4 is a chart summarizing DEP-FFF separation data for various cell types.

FIG. 5 is a picture showing a spiral electrode array that may be used to focus cells by twDEP.

FIGS. 6A-6B are charts showing field/frequency bursting characteristics of (A) T-lymphocytes, and (B) MDA-MB-435 breast cancer cells.

FIG. 7 is a graph showing magnetic field strength emerging from two opposing magnets.

FIG. 8 is a flow chart illustrating functional stages of a device for cell isolation and analysis.

FIG. 9 is a schematic of an integrated fluidic system, including a prefilter stage, a separator stage, and an isolator and analysis stage.

FIG. 10 is a schematic showing a short section of a DEP-MAP-FFF chamber.

FIG. 11 is an end view of a magnetophoresis assembly. The magnets are SmCo or NdFeB. The separation chamber sits in the magnetic flux gradient just above the sintered iron spheres. Sintered iron spheres may be replaced by iron wedges or filaments to produce different desired flux gradient characteristics.

FIG. 12 is a schematic of one embodiment of the integrated fluidic system, including a prefilter stage, a separator stage, and an isolator and analysis stage that includes a programmable fluidic processor.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0013] The presently disclosed systems, methods and apparatuses provide many advantages (a few of which are the following). They permit for cell pre-filtering that may be used to separate tumor cells from peripheral blood mono-nuclear cells (PBMNCs). They allow for Dielectrophoretic-magnetophoretic field-flow fractionation (DEP-MAP-FFF), allowing for combined dielectrophoretic and immunomagnetic cell separation. They allow for the dielectric indexing of

beads, the linkage of antibody and oligonucleotide probes to bead surfaces, and for the simultaneous assays for two molecular markers using a mixture of two different bead/probe types. They allow for the quantification of the association of targets with the beads and identifications of the bead types by dielectric measurements using impedance sensing methods. They allow for DEP-MAP-FFF fractionation of cells according to their surface receptor concentrations. They
5 allow for DEP focusing of samples using swept frequency traveling fields applied to spiral electrode arrays that can be used to concentrate isolated cell fractions to ~ 10⁹ cells/ml. They allow for the electro-mediated bursting of cells. They also allow for the mixtures of different bead/probe combinations to be used to perform parallel assays with dielectric indexing.

10 [0014] Areas that may benefit from this technology include, but are not limited to: blood and urine assays, pathogen detection, pollution monitoring, water monitoring, fertilizer analysis, the detection of chemical and biological warfare agents, food pathogen detection, quality control and blending, massively parallel molecular biological protocols, genetic engineering, oncogene detection, and pharmaceutical development and testing.

15 [0015] Because the present disclosure deals, in part, with the combination of a number of technologies that may be discussed separately, it is useful to begin the discussion with some theoretical underpinnings and considerations relating to some of the individual techniques disclosed herein. In the Examples section, discussion will focus more upon the combination of the techniques to form systems and apparatuses (and associated methodology) according to embodiments of this disclosure.

20 [0016] Certain techniques of this disclosure use molecular recognition and sensing elements that are attached to bead carriers so that a new aliquot of sensitized beads can be used for each and every assay. By disposing of the beads afterwards, by running a "blank" between each sample, and by allowing for cleaning cycles, calibration issues can be addressed and the absence of carryover and cross-contamination can be verified.

25 [0017] Placing biologically active components on beads also means that a single, fluidic device may be applied to a wide range of sample preparation and molecular analysis problems by using different bead/probe combinations. Finally, because no biological components need be attached to fixed surfaces within the device of one embodiment, those surfaces may be PTFE coated, for example, to reduce biomolecular adhesion and carryover issues. It follows that the use of beads enhances the potential applicability of the technology by allowing a single device to have multiple applications.

30 [0018] Although molecular amplification techniques enjoy widespread use, methods such as PCR have drawbacks including sensitivities to hard-to-control factors that can render them only marginally quantitative. Furthermore, molecular amplification bathes the reaction surfaces with high concentrations of the molecules to be detected. The resultant carryover problem is so severe that all wetted surfaces in molecular amplification experiments are typically made disposable. For these reasons, this disclosure avoids direct molecular amplification steps in designing reusable devices and focuses on detecting small numbers of molecules trapped directly on beads. Nevertheless, having the benefit of this disclosure, those having skill in the art recognize that the bead-based indexing technology described here is also compatible with molecular amplification protocols should they be required.

35 [0019] Any *in-situ* hybridization assay may be adapted to operate on the surface of a carrier bead including methods for detecting DNA, RNA and proteins. In this disclosure, the established body of hybridization and immuno-fluorescent molecular techniques may be used along with new methods for indexing bead carriers so that individual bead types within a complex mixture of bead types are identifiable, amenable to selective manipulation, and, if desired, to isolation.
40 Assays using dielectrically-engineered beads require minimal quantities of sample. For example, a bead of about 5 µm diameter has the relatively large surface area of approximately 78 µm² yet occupies a volume of only 65 fL, about 1/15 that of a typical tumor cell. 100 tumor cells and 250 beads comprised of 10 different bead types may be packed into spherical region of 50 µm diameter using DEP-mediated focusing. This is the equivalent of almost 10⁹ cells/ml held in contact with 2 x 10⁹ beads/ml carrying the molecular probes. The time for hybridization of target mRNA's to cDNA probes on magnetic bead surfaces has been shown to be just a few minutes in concentrated cell lysates; therefore, the bead-based approach of this disclosure may enable rapid assays for molecular markers in an integrated system.

45 [0020] The bead-based, dielectric indexing technology of this disclosure is not meant to replace large gene-chip array methods designed for massively parallel analysis of the expression of 10,000 or more genes. Those methods permit the identification, in the first place, of key markers of specific cellular events. Instead, this disclosure represents a technology in which a reduced panel of 10 or so key molecular markers may be selected from a library of available markers for the purpose of screening for specific subsets of suspected disease states. By combining sample-preparation and molecular analysis into a single, automated process, this system allows the exploitation of gene-chip-derived molecular epidemiological data and renders it accessible to a wide population.

55 [0021] This disclosure addresses the isolation of suspect cells from mixed cell suspensions and the manipulation of mixtures of dielectrically indexed beads, all in an integrated device. Achieving these steps ultimately depends upon ways of moving matter with respect to the solution that suspends it, a problem to which dielectrophoresis, or another suitable manipulation force, is ideally suited.

[0022] Dielectrophoresis (DEP) is the movement of a material or an object caused by a spatially non-uniform electrical field. Completely distinct from the well-known phenomenon of electrophoresis, DEP only arises when the object has a different tendency to become electrically polarized relative to its surroundings. If the object is more polarizable than its surroundings, it will be pulled-towards higher field regions ("positive DEP"); conversely it will be repelled towards weak field regions ("negative DEP") if it is less polarizable. Positive DEP is known to most of us as the attraction of uncharged bits of paper to a charged plastic comb. Magnetophoresis is the magnetic analog of dielectrophoresis, the collection of magnetically polarizable particles in a spatially non-uniform magnetic field. This force is responsible for the familiar collection of iron filings at the fringing fields at the edges of a magnetic pole. Far from being restricted to electrostatic fields, DEP also occurs in alternating (AC) fields even at optical frequencies. An example is when a laser tweezers is used to trap a cell having a higher refractive index (larger electronic polarizability) than its suspending medium at the high field gradient focal region of the laser beam. (There is also a second, light pressure term in this extreme case). At lower frequencies DEP can be used to impose forces on cells that depend on their low-frequency spectral properties. Differences in these spectral properties can be exploited to impose different or even opposing forces on different cell types in a cell mixture. For techniques of this disclosure, relatively low frequencies may be used, from about 10 kHz to about 10 MHz, at which cell membrane and bead coating properties dominate the particle dielectric properties.

[0023] The essential characteristic of DEP is the movement of objects with respect to their suspending medium. For example, objects can be concentrated to a focal point by negative DEP and/or trapped by positive DEP. In addition, different particle types can be moved apart from one another in three dimensions under appropriate field conditions. These basic manipulations can be used to sort, isolate, and trap cells and beads, and to change the reagents in which they are suspended.

[0024] Of particular relevance to this disclosure is the extensive DEP work on normal and cancer cells in which the inventors and others have shown that different cell types have distinct dielectrophoretic fingerprints and may be used (in accordance with embodiments disclosed herein) to characterize, manipulate, fractionate, isolate, trap, and selectively burst them.

[0025] Summarizing, DEP is a force that:

1. arises when a particle having dielectric properties distinct from its carrier medium is subjected to a spatially non-uniform electrical field anywhere from DC to optical frequencies;
2. in complete contrast to electrophoresis, completely ignores any net charge on the particle (this is a critical consideration when performing assays with highly charged biomolecules such as nucleic acids);
3. can be used to trap, focus, fractionate and isolate cells;
4. depends specifically on the dielectric fingerprint of each cell type. In principle, DEP can be used to exploit any spectral differences between cells but this disclosure focuses on low frequency differences dominated by plasma membrane morphological characteristics;
5. can be produced by an AC electrical field that typically has a frequency between 10 kHz and 1 MHz for cell isolation experiments. No electrolysis occurs at these frequencies and cells are not damaged unless the field is deliberately increased above a high threshold magnitude to achieve controlled cell bursting;
6. can be produced by an array of micro electrodes that are inexpensive to fabricate according to methods known in the art;
7. can be produced at AC frequencies even if the electrodes carry a thin coating of PTFE or other insulator;
8. is controlled via the frequency and/or voltage of the signal applied to the electrodes. The electronics are straightforward, can be incorporated in a box the size of a pocket calculator, are inexpensive, and can be kept separate as is all known in the art so that DEP chambers may be disposable while the electronics are retained;
9. is ideal for meso- and microfluidic-scale applications because electrodes can line the floor and/or walls of fluidic channels and chambers;
10. allows cells, beads, or other targets to be selectively manipulated within their carrier medium or held in place while a new carrier medium is washed over them.

[0026] In one embodiment, high discrimination sample preparation of suspect tumor subpopulations is accomplished through a separation technique called hyperlayer field-flow fractionation. The underlying principle is straightforward: the velocity of fluid flowing through a flat channel increases from zero at the floor and ceiling to a maximum at the center. If different cell types are positioned at different characteristic heights above the channel floor then they will be carried at different velocities by the fluid and separated as the cell mixture travels through the channel. The different types can then be isolated and trapped as they emerge from the far end of the channel. Separation does not depend on the interaction of cells with any material other than the carrier fluid, reducing non-specific binding, carryover, and contamination effects that are inherent in chromatographic methods, for example.

[0027] To position different cell types characteristically in the separation channel, one may balance dielectrophoretic

and gravitational forces on cells. Additionally, magnetophoretic forces may be used as well for positioning cells if desired. In this way, immunomagnetic labeling can be used as an additional feature to discriminate between different cell types. The DEP-MAP-FFF method is equally applicable to cells, which have their own intrinsic dielectric properties, and to beads that can act as molecular marker carriers. When a cell subpopulation has intrinsic dielectric differences that distinguish it from other cell types in a mixture, it is not necessary to use magnetic labeling and the method may revert to a DEP-FFF scheme.

[0028] The continuous MAP-sorting of immunomagnetically labeled cells in a laminar flow profile subjected to a quadrupole magnetic field configuration has been demonstrated. While the sorting of cells according to surface receptor density was achieved, the method has the disadvantage that the MAP force is unbalanced. Consequently, separation is flow-rate dependent. Furthermore, heavily labeled cells may collide with the sides of the flow chamber only to become trapped or to suffer remixing with other cell types. The DEP-MAP-FFF design of the present disclosure, however, balances opposing DEP and MAP forces to place cells in equilibrium positions in the flow profile. In this way, the pitfalls of unbalanced forces, which are likely to be of even greater concern when sorting inherently inhomogeneous tumor cell subpopulations, may be avoided.

[0029] In addition to cell sorting, DEP may be used to prefilter cells when large numbers of cells need to be processed, to trap cells after they emerge from the DEP-MAP-FFF separator, to concentrate the cell isolates and beads, to lyse the cells, and to hold beads in place while reagents are changed in molecular analysis protocols. In this way, dielectrophoresis provides for the ability to realize an automated device that will integrate a sample prefilter, a DEP-MAP-FFF separator, a cell fraction isolation and lysis stage, and a molecular analysis stage.

Sample Preconcentration.

[0030] In one embodiment, a DEP-MAP-FFF system may take a sample of about 20 μL of cell suspension containing a maximum of 2×10^5 cells when performing high resolution separations. A lower detection limit of 20 cancer cells in the molecular analysis stage requires an incidence of 1 or more cancer cells per 1000 normal cells. While this level of discrimination is adequate for biopsy samples of putatively tumorous tissue, in other applications, such as the detection of residual disease, of metastatic cells in bone marrow harvests, or of micrometastases in sentinel lymph nodes, the goal is to detect 1 tumor cell per 10^6 or more normal nucleated cells. To provide 20 tumor cells for analysis in such applications, there is the need to sort $> 2 \times 10^7$ normal cells, a number that far exceeds the capacity of DEP-MAP-FFF separator stage because to achieve high discrimination this stage needs to operate at cell concentrations where cell-cell interactions are negligible.

[0031] To sort high numbers of cells, a stage that will execute a DEP prefiltering step for suspect cancer cells may therefore be needed. While prefiltering does not provide a pure population of suspect cells, it does provide a sample that is suitable for the DEP-MAP-FFF stage of the device (which is explained and illustrated, in one embodiment, in the Examples section of this disclosure). In one embodiment, the prefilter may process $\sim 20 \times 10^6$ cells and extract $\sim 2 \times 10^5$ cells enriched in the suspect cell subpopulation. Those 2×10^5 cells may then be routed to a high discrimination DEP-MAP-FFF separator stage. If the lower limit of molecular analysis in the last stage of the integrated device is 20 cancer cells, then the integrated device may achieve a detection limit of 1 cancer cell per 10^6 starting nucleated cells.

Dielectrophoresis

[0032] It has been shown that the DEP force acting on a particle due to an imposed electrical field, $\vec{E}(\omega)$, can be written as

$$\langle \bar{F}(t) \rangle = 2\pi\epsilon_m r^3 (\text{Re}(f_{CM}(\omega))\nabla E(rms)^2 + \text{Im}(f_{CM}(\omega))(E_{x0}^2\nabla\varphi_x + E_{y0}^2\nabla\varphi_y + E_{z0}^2\nabla\varphi_z)) \quad (1)$$

where

$$f_{CM}(\epsilon_p, \epsilon_m, \omega) = \frac{\epsilon_p(\omega) - \epsilon_m(\omega)}{\epsilon_p(\omega) + 2\epsilon_m(\omega)}. \quad (2)$$

is the Clausius-Mossotti factor that embodies the frequency-dependent dielectric properties $\epsilon_p(\omega)$ and $\epsilon_m(\omega)$ of the particle and its suspending medium, respectively. ω is the angular frequency and $E(rms)$ is the rms value of the applied

electric field. E_{i0} and ϕ_i ($i=x; y; z$) are the magnitudes and phases, respectively, of the field components in the principal axis directions. Equation (1), which is sufficient for the present discussion, shows there are two independent force contributions to DEP motion:

- 5 (i) *A field inhomogeneity component*: the left hand term depends on the *real* (in-phase, or capacitative) component $\text{Re}(f_{CM})$ of the induced dipole moment in the particle and the spatial nonuniformity, $\nabla E(\text{rms})^2$, of the field magnitude. This force pushes particles towards strong or weak field regions, depending upon whether $\text{Re}(f_{CM})$ is positive or negative. This is the DEP force that allows cells to be attracted or repelled from electrode edges. It is the only DEP force component that can act when an electrode array is energized by single or dual phase signals.
- 10 (ii) *A traveling field component*: the right hand term depends on the *imaginary* (out-of-phase, or lossy) component $\text{Im}(f_{CM})$ of the induced dipole moment and the spatial nonuniformity ($\nabla\phi_x$, $\nabla\phi_y$, and $\nabla\phi_z$) of the field *phase*. This force pushes the particle in the same or the opposite direction to which the field is traveling depending on the sign of $\text{Im}(f_{CM})$. It allows cells to be swept along by an electric field that travels over an electrode array. At least three excitation phases must be provided for this force to arise.
- 15

[0033] These force components act independently but, by appropriate electrode array design, can be applied simultaneously to levitate cells above an electrode array while moving them over it, for example.

20 Cell Dielectric Properties

[0034] At low frequencies cells exhibit negative DEP (repulsion from electrode tips) but at higher frequencies, above their so-called DEP crossover frequencies, they exhibit positive DEP (attraction towards electrode tips). Different cell types have different crossover frequencies. At frequencies between about 10^4 and 3×10^4 Hz breast cancer cells will experience positive DEP trapping while blood cells will experience negative DEP repulsion. These dielectric differences between the cancer and blood cell types can be used as a basis for cell identification, discrimination and separation. Cell sizes, cell compositions, and especially cell membrane morphologies all contribute to the dielectric differences between the cells; i.e. different cells have different dielectric phenotypes.

[0035] The inventors have found that the dielectric phenotype of every transformed cell type they have examined is significantly different from that of a more normal cell of origin, or from the same cell type following induced differentiation. This results from greater cell surface morphological complexity and a correspondingly higher membrane capacitance in the transformed cell types. Furthermore, tumor cells are normally much larger than blood cells. The effect of these combined differences is that the dielectric properties of transformed cells differ very significantly from normal blood cells. Of particular relevance to this disclosure, the inventors have measured the DEP crossover frequencies of 9 human cancers comprising 5 human breast cancer cell lines, an ascites sample taken from a patient with breast cancer, and two colon cancer cell lines. The DEP crossover frequencies of these cancer cell types suspended in solutions of 100 mS/m conductivity are shown in FIG. 1 in comparison with data for normal peripheral blood mononuclear cell types. The tumor cells all exhibit much lower crossover frequencies. These differences may be exploited for isolating populations of suspect cells from PBMNCs and lymph cell dispersions.

40 Prefiltering by DEP Trapping of Cells

[0036] Exploitation of dielectric differences for cell separation may be accomplished in several ways. The simplest though least discriminating method is to apply a frequency that repels one cell type from one or more electrodes by negative DEP while attracting and trapping a different cell type by positive DEP. FIG. 2A shows a mixture of MDA-MB-231 human breast cancer cells and human peripheral blood. The larger breast cancer cells, about $12 \mu\text{m}$ in diameter, are readily identifiable. In FIG. 2B, a 2.5×10^4 Hz AC signal has been applied between neighboring gold electrodes (dark patterns) and fluid flow has been started from left to right. The human breast cancer cells are attracted to the electrode tips and trapped (FIG 2B&C). Blood cells, on the other hand, are repelled from the electrodes and carried off by the fluid. They emerge downstream, where no cell mixture was loaded, free of cancer cells, (FIG. 2D). This DEP trapping approach works well when there are large differences in the dielectric properties of target cells and other cell types in the starting mixture. For example, the inventors have demonstrated that it is possible to recover 100% of human breast tumor cells from PBMNCs even at the most dilute concentration tested in preliminary experiments, one tumor cell per 3×10^5 PBMNCs.

[0037] After flushing out the blood cells, tumor cells may be recovered by lowering the frequency below 10 kHz causing them to be repelled from the electrodes by negative DEP and released from the chamber. The inventors have found that some normal cells may be associated with the tumor cells during the trapping phase such that while recovery efficiency may be extremely good, purity may not be so good. It should be noted that at higher applied frequencies

(200kHz or more) all viable cells have been found to become trapped by positive DEP regardless of type. Therefore, DEP may be used quite generally to immobilize cells within a stream of reagents without regard to cell type if required.

[0038] In applications involving rare cancer cells, a prefilter system may be used having a surface area of about 60cm² over which suspensions of nucleated cells can be passed at the rate of about 3.6x10⁶ cells/min. This may be operated for about 6 minutes with suspensions of cells from lymph nodes and whole blood to screen 20 x 10⁶ nucleated cells for the presence of tumor cells. Suspect cells, at a purity of >0.1%, may then be passed for high discrimination separation by the DEP-MAP-FFF in and, after subsequent isolation, for downstream molecular analysis in the integrated device (discussed in more detail in the Examples section of this disclosure).

10 DEP-FFF Separation

[0039] To allow high discrimination separation of tumor cells from biopsy samples or from lymph node or blood cell samples prefiltered by DEP trapping, a fractionation method termed DEP-MAP-FFF may be used. Such a method may also use immunomagnetic capabilities when needed. Instead of trapping target cells, DEP-FFF uses parallel electrodes without castellated edges to levitate cells above the electrode plane using fringing fields. The strength and inhomogeneity of the electrical field decreases with increasing height above the electrode plane and the DEP force on cells falls exponentially with height. If a frequency for which cells experience negative DEP is applied to the electrode array, cells will be levitated to a height at which the repulsive DEP force balances the sedimentation force. Cells having differences in density and/or dielectric properties will therefore be levitated to characteristic heights as illustrated in FIG. 3. This equilibrium height is given by

$$25 \quad h_{eq} = \frac{d}{2\pi} \ln \left\{ \frac{3\epsilon_m U^2 A p}{2(\rho_c - \rho_m)g} \operatorname{Re}(f_{eq}) \right\} \quad (3)$$

for a parallel electrode geometry, where U is the electrical potential applied to the electrode array, A is a geometrical term, p is the proportion of the applied field unscreened by electrode polarization ($p \sim 1$ at frequencies > 50 kHz), and $(\rho_c - \rho_m)g$ is the sedimentation force.

[0040] To exploit this equilibrium levitation effect for cell fractionation, fluid flow is initiated in the channel. Fluid flows through the channel in a parabolic profile - slowest at the chamber top and bottom walls, and fastest in the middle (at about half height according to one embodiment). The velocity at height h_{eq} , is given by

$$35 \quad v_p = \langle v \rangle \frac{h_{eq}}{H} \left(1 - \frac{h_{eq}}{H} \right), \quad (4)$$

where H is the chamber height and $\langle v \rangle$ is the mean fluid velocity. The fluid will then carry cells at a velocity corresponding to their levitation height. Mixed cell types starting at one end of a long chamber will therefore be separated according to their dielectric and density properties.

[0041] The family of techniques that exploits hydrodynamic flow profiles for separation is termed field-flow fractionation (FFF); hence the inventors term this method DEP-FFF. The discriminating power of DEP-FFF is extremely high in the frequency range where the cell dielectrophoretic force approaches zero (i.e. near the crossover frequencies shown in FIG. 1). Less discriminating power can be selected electrically by employing a lower frequency or by using modulated frequencies.

[0042] The inventors have made several DEP-FFF separators ranging in size from about 45 cm x 2 cm to the size of a microscope slide (see section below concerning microfabrication). With the benefit of the present disclosure, those having skill in the art recognize that other sizes may be used as well. DEP-FFF separation normally take from 4 to 15 minutes to complete, but this time may vary significantly depending on the size of the device and other parameters such as sample size. For different separation times for different cell types, under different experimental parameters, see FIG. 4.

[0043] In one embodiment, a modified form of DEP-FFF may be employed in which an additional vertical force component is added that depends on immunomagnetic labeling of the cells. This may address potential concerns that some tumor cell types might not have intrinsic dielectric properties like those shown in FIG. 1 that permit their separation from normal cells by DEP-FFF alone. The inventors feel that exploitation of cell intrinsic properties, when possible, may be more desirable than requiring a labeling step; therefore, they have designed DEP-MAP-FFF separators so that exploitation of immunomagnetic labeling is an available, though non-essential, option: in the absence of immunomag-

netic labeling, the device may function as a DEP-FFF separator that can discriminate cells by dielectric properties alone.

Magitetophoresis (MAP)

- 5 [0044] A particle of volume v and magnetic permeability μ_p subjected to an inhomogeneous magnetic field will experience a MAP force that is the magnetic analog of the DEP force given in equation (1)

$$\bar{F}_{MAP} = 2\pi\mu_s r^3 k_{CM}(\mu_s, \mu_p, \omega_H) \bar{B} \cdot \nabla \bar{B} \quad (5)$$

10

Here, μ_s and μ_p are the magnetic permeability of the suspending medium and particle, respectively, R is the particle radius and, $k_{CM}(\mu_s, \mu_p, \omega_H)$ is the Magnetic Clausius-Mossotti factor describing the magnetic polarizability of the particle with respect to its suspending medium. In the static fields typically used for MAP cell sorting, ω_H , the frequency of the applied magnetic field, has the value 0 and μ_s and μ_p become static magnetic permeability parameters. Furthermore, the magnetic permeability of the aqueous suspension in an immunomagnetic labeling experiment can be approximated as that of free space and the net polarizability of a labeled cell can be assumed to result from the combined effect of n identical labels that are bound to it. Finally, for a fixed geometry, the magnetic field gradient may be written as a geometry term G_{MAP} times the applied magnetic field strength, B_0 . Hence, in a biological labeling experiment we may simplify the MAP force equation to

15

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$$\bar{F}_{MAP} = n\varphi G_{MAP} B_0^2 \quad (6)$$

25 where φ is a constant for a given magnetic label type. This is the fundamental equation that determines magnetic capture of cells in MACS; however, the goal of the present disclosure is not to magnetically trap cells. By appropriate design of the magnetic elements that create the magnetic field and its inhomogeneity characteristics embodied in G_{MAP} , a MAP force may be provided that is essentially constant throughout a separation chamber and directed towards the chamber floor.

30 [0045] We indicated earlier that the DEP force above a parallel electrode array falls off exponentially with height h as $F_{DEP} = F_{DEP,0} e^{-h/h_{DEP}}$. When the electrical field conditions are chosen to provide repulsive DEP, as in DEP-FFF, the MAP force will pull an immunomagnetically labeled cell toward the electrode plane until the sum of the downward MAP and sedimentation forces are balanced by the levitating DEP force. Writing the electrical field gradient in terms of an electrode geometry term G_{DEP} and the applied RMS voltage V_0 applied to the electrode array, the balance of forces 35 that determines the particle equilibrium height will be given by eq. 7 below:

35

$$\bar{F}_{MAP} + \bar{F}_{gravity} + \bar{F}_{DEP} = n\varphi G_{MAP} B_0^2 + [n(\rho_p - \rho_s) + nm_{label}]g + 2\pi\epsilon_s R^3 f_{CM}(\epsilon_m, \epsilon_p, \omega) G_{DEP} V_0^2 e^{-h/h_{DEP}} = 0$$

40

where m_{label} is the mass of each immunomagnetic label. If the magnetic labeling is negligible ($n \rightarrow 0$), this equation reduces to that given earlier for plain DEP-FFF. On the other hand, if magnetic labeling dominates the downward force then the decrease in h becomes approximately proportional to the logarithm of the number n of magnetic labels attached to the cell. Since in this context "dominate" means to provide a MAP force significantly in excess of the small cell sedimentation force, it will be appreciated that much smaller magnetic forces are needed in DEP-MAP-FFF than for magnetic trapping against a flow stream as used in MACS.

45 [0046] Note also that V_0 can always be chosen to ensure that no cells are pulled all the way to the chamber floor. Because, according to one embodiment, cells are separated in a FFF scheme according to their characteristic heights h in the fluid flow profile, one may separate them according to the extent of immunomagnetic labeling and, as is familiar 50 in fluorescently-activated cell sorting (FACS), the logarithmic relationship may be very convenient for ensuring a good dynamic range when sorting different classes of cells. Therefore, when needed, MAP provides an ideal additional level of discrimination for sorting suspect-tumor cell-subpopulations by, for-example, epithelial surface markers or receptors such as for EGF.

55 *DEP-Mediated Cell Focusing*

[0047] Cells can be manipulated simultaneously by DEP, which attracts or repels them from electrode edges, and

twDEP, which transports them parallel to the plane of the electrodes. A spiral electrode configuration may be used to exploit these effects simultaneously for concentrating cells and achieving electrically stimulated cell lysis. The spiral array in one embodiment includes four parallel electrode elements that are energized by signals of the same frequency but phases of 0°, 90°, 180°, and 270° to create a concentric traveling field that sweeps towards the center of the spiral. Excitation by phases 0°, 270°, 180°, and 90° results in a field that sweeps outward towards the periphery of the spiral. Signals of 0°, 180°, 0°, and 180° phases produce a stationary field pattern that can be used for DEP trapping, levitation, or, at very high field strengths, cell bursting.

[0048] An example of cell trapping and focusing is shown in FIG. 5 where HL-60 human promyelocytic leukemia cells have been focused from a scattered state to the center of a spiral in about 15 seconds. In one embodiment, the spiral arms of the electrode array may be extended until they almost touch at the center of the spiral allowing greatly increased cell concentrations to be achieved. The inventors have applied this technique to trap and focus murine erythroleukemia and human breast cancer cell lines from a flow stream, and separate breast and leukemia cells from blood cells. Also the inventors have successfully separated erythrocytes parasitized by the malarial agent *Plasmodium falciparum* from their uninfected counterparts with this technique.

[0049] In one embodiment, five spiral array segments may be used to trap cell subpopulations as they emerge at different times from a DEP-MAP-FFF separator stage of an integrated device. By injecting assay beads into the stream of cells as they emerge from the separator and before they are trapped, and by then applying a swept field to the spiral electrodes, cells and beads may be focused to the center concurrently to form a highly concentrated mixture.

20 *Electro-Mediated Lysis of Cells*

[0050] Once a target cell population has been successfully isolated, subsequent molecular analyses normally require that the cells be disrupted to release intracellular proteins, RNA, and DNA. Approaches to this include exposure to detergents or other lysing reagents. Although these methods can be used in systems and devices disclosed herein, cells may be lysed electrically using large AC electrical fields. DEP manipulations typically involve local electrical fields less than 10^4 V/m and the inventors have shown that cells can sustain prolonged (40 minutes and longer) exposure to such fields without loss of viability or activity. Depending on the electrode geometry, voltages of the order of 1 V RMS are used to achieve this.

[0051] However, higher AC voltages may be applied to create fields that can burst cells. Depending on the cell type, at about 5×10^4 V/m, temporary membrane electropermeabilization occurs, and this can be used to load reagents into cells. Above about 2×10^5 V/m, instantaneous destruction of the cell membranes occurs. The inventors have found that different cell types have characteristically different susceptibilities to destruction. FIG. 6A illustrates the field intensity vs. frequency dependency for the disruption of human T-lymphocytes and FIG. 6B shows results for human MDA-MB-435 breast cancer cells. Clearly the cells burst in characteristic, and distinct, frequency and field ranges. A useful feature is the ability to select electrically whether to reversibly permeabilize or totally disrupt all, or select subpopulations, of cells that have been trapped on an isolation electrode.

[0052] In one embodiment, electro-mediated cell lysis may be utilized at the center of the spiral isolation segments to release molecular species from target cells into the immediate vicinity of the assay beads mixed and concentrated with them.

40 *Microfabrication*

[0053] Electrode arrays for use in, for instance, a separation according to embodiments of the present disclosure may be made by microlithography as is known in the art. The inventors have built DEP chambers and separators over a wide range of sizes from about 200 μm - 45 cm with capacities of 10 μL to 4 mL. The use of silicon and glass and micromachining methods may be used for cases where integrated electronics and sensor capabilities are required that other fabrication methods cannot provide. In other cases, a combination of flat glass and injection-molded polymers may be used to fabricate the devices disclosed herein by methods known in the art. Small devices may be made by silicon and glass micromachining, and can be reproduced by single layer lithography on a flat glass substrate (for the electrodes) with all fluidic channels molded into a clear polydimethylsiloxane (PDMS) top. Molding PDMS has been suggested as a much more cost effective approach than micromachining glass and silicon; it comes as a clear liquid that can be cast or injected into a mold. Devices of the present disclosure may be designed to handle not only small (about 20 μL) samples but also larger volumes (~ 10 mL or more). To accomplish this, a microfluidic front-end is clearly unsuitable because it would be unable to process large samples at reasonable rates. In one embodiment, the sample may be enriched as it passes through the device and to simultaneously reduce its volume. In this way a microfluidic stage, with its advantages of small sample requirements and rapid processing capabilities, may be seamlessly interfaced to the macroscopic world to complete the molecular analysis.

Magnetic Field Generation

[0054] The MAP force to be used in conjunction with DEP-FFF requires a magnet having rather unusual properties, namely the product of the magnetic field strength and its inhomogeneity need to be effectively constant over the entire length of the separator. To achieve this, one may use several flat magnets of SnCo or NdFeB materials placed a parallel configuration in an opposing pole orientation. FIG. 7 shows two magnets in this configuration. The field lines experience compression in the space between the opposing poles and emerge in a relatively homogeneous distribution. Controlled inhomogeneity in the field may be created by using a composite material made of sintered iron spheres in the field path.

[0055] The field strength and homogeneity (in the absence of the sintered iron elements) has been tested for two 6 mm thick SnCo magnets having 25 mm x 25 mm pole faces and a "free field" of 0.22 T in air. The field of the opposing pole configuration was measured with a directional Hall probe. Field strengths in excess of 0.4 T were measured (FIG. 7) for pole spacings of 4 mm or less and the horizontal field component was below 5%. Based upon the inventors' measurements of the magnetic fields used in small MACS separators, these intensities are more than sufficient to achieve magnetic positioning of immunomagnetically-labeled cells in DEP-MAP-FFF.

[0056] The following examples are included not for limitation but, rather, to demonstrate specific embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute specific modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 - Design Issues

[0057] In one embodiment, the present disclosure is directed to an integrated fluidic device able to sort, isolate and burst target cells from clinically relevant samples and to execute molecular marker assays on them rapidly and automatically. FIG. 8 shows a functional block diagram of a complete integrated device and FIG. 9 shows a design for the system.

Prefilter

[0058] FIG. 9 shows the design of the prefilter and DEP-MAP-FFF cell fractionation stages of the device. The prefilter is essentially a scaled-up version of a DEP cell trapping device. Its purpose is to cope with the huge numbers of cells that need to be sorted in rare cell detection applications. It is aimed at capturing all cancer suspect cells even at the expense of retaining some normal cells. The scaled-up prefilter is designed, in one embodiment, to process a sample of ≤ 10 ml volume containing $\leq 2 \times 10^7$ cells in ≤ 10 minutes at a maximum rate of 3.6×10^6 cells per minute. It is designed to extract from that sample suspect cells that will be passed to the second, high discrimination cell fractionation stage (discussed below).

[0059] Samples may be exemplified by peripheral blood from which erythrocytes have been lysed, dispersions of lymph node tissue, or dispersed biopsy cells. To achieve sorting in ≤ 10 minutes, the prefilter may sort $\leq 1000 \mu\text{L}$ of cell suspension per minute. This may be accomplished by a DEP trapping array lining the floor of a chamber 20 mm wide, 400 μm high and 30cm long. These dimensions ensure that (1) suspect cancer cells in the mixture are guaranteed sufficient time when traversing the chamber to sediment close enough to the DEP electrode array to be trapped by an applied 50 kHz field while normal blood cells are repelled; (2) hydrodynamic forces experienced by trapped cells remain sufficiently weak not to dislodge them from the electrode array; and, (3) cell density remains sufficiently low that suspect cells are not knocked away from electrodes by collisions with an over-abundance of other cells.

[0060] After processing the starting suspension, clean eluate may be passed through the prefilter at about $400 \mu\text{L}/\text{min}$ to wash away remaining untrapped cells. During this rinsing phase, the DEP prefilter trapping electrode may be de-energized while the secondary trapping stage remains energized. Suspect cells in the prefilter stage may be released and carried to the secondary trap. This consolidation of trapped cells is made possible by the removal of the large concentration of normal cells from the system. Throughout these early phases, emerging eluate may be sent to waste.

[0061] After-the-consolidation-step, the secondary trapping stage may contain the suspect cancer cells together with some entrapped blood cells. Based on the inventors' experience, this stage is expected to collect a total of no more than 2×10^5 "suspect" cells at this stage. The suspect cells may include a limited number of monocytes, some macrophages, and any other large circulating cells including all of the true cancer cells. This number of cells is ideal for high discrimination sorting by DEP-MAP-FFF because the cell density has been sufficiently reduced for cell-cell interactions to be ignored. A major advantage of the prefilter design is its relative tolerance of such cell-cell interactions.

Magnetic Antibody Labeling

[0062] The cells in the secondary trapping stage may be incubated with magnetically labeled antibodies if MAP separation is to be utilized in the next step of cell isolation. Also, fluorescent antibodies, appropriate for surface marker detection much further downstream in the device, may be added at this point. To accomplish labeling, antibodies may be injected into the port provided for this purpose while the cells are held in place on the electrode by DEP forces from a field of, in embodiment, about 250 kHz. Once fluid flow has stopped, a DEP field of about 3 V peak-peak may be alternated between about 10 kHz and about 250 kHz at about 10 second intervals to alternately levitate and trap the cells, gently stirring them with the antibodies. Following incubation, the DEP field may be switched to about 250 kHz to trap the cells while the antibodies are washed away and the cells are rinsed with fresh buffer.

DEP-MAP-FFF Injection

[0063] Following the optional antibody-labeling steps, a 0.5 V, 10 kHz signal may be applied to release the suspect cells from the secondary trapping electrode without levitating them. Fluid flow may be initiated in the prefilter stage and the cells may be flushed into the DEP-MAP-FFF stage via the fluid splitter. Because of the dimensions of the chambers and the splitter position, the suspect cells may be carried into the DEP-MAP-FFF stage in 20 μ L of eluate. A syringe pump at the end of the DEP-MAP-FFF stage may be used to control the sample flow.

[0064] In applications such as analysis of fine needle aspiration biopsy samples, the starting cell count may be about 2×10^5 cells or less, and the prefiltering step becomes superfluous because the DEP-MAP-FFF fractionator can handle such small samples without undesirable cell-cell interactions. Such samples may be injected into the preconcentrated sample loading port at the concentrator injection stage for the optional antibody labeling steps and thence directly into the DEP-MAP-FFF sorter.

DEP-MAP-FFF Fractionation

[0065] During and after injection of the cell sample from the prefilter stage, the DEP electrode array in the DEP-MAP-FFF separator stage may be energized with a frequency appropriate for separation, typically in the 20-80 kHz range. With flow stopped, cells may be allowed sufficient time to reach equilibrium heights at which the magnetic, DEP and gravitational force fields acting on them are balanced. Based on DEP-FFF experiments this so-called relaxation time need not exceed five minutes. Following relaxation, fluid flow through the DEP-MAP-FFF stage may be initiated and cells may be carried through the chamber at characteristic velocities in accordance with their positions in the parabolic flow profile controlled by the balance of DEP, MAP and gravitational forces. Based on DEP-FFF experiments, this separation step should take, in one embodiment, 12 minutes or less.

Trapping of Cell Fractions

[0066] As at the interface of the prefilter and DEP-MAP-FFF stages, a split flow may be used between the DEP-MAP-FFF stage and the isolator and analysis stage so that only flow close to the bottom of the separator, in which cells may emerge, is passed through. The remaining eluate may be extracted from above and sent to waste. A controlled flow of analysis beads may be injected into the flow stream as it emerges from the DEP-MAP-FFF separator and enters the isolation and analysis stage. This may mix analysis beads with the emerging cell fractions.

[0067] The cell isolation stage may be divided into 5 separate electrode array segments, each capable of trapping and concentrating a separate fraction of cells that emerges from the separator. Before any cells have emerged, a non-traveling 10kHz field may energize the first 4 segments of the isolation stage. This may levitate both cells and beads by negative DEP and prevent them from settling on those segments. However, the fifth segment may energized at 500 kHz, a frequency at which all cell types and the beads may become trapped. Therefore, the first cells to emerge, and the beads mixed with them, may be carried across the first 4 segments and be trapped on the fifth by positive DEP. After an appropriate time span to isolate the first fraction of cells on the fifth segment, the 4th segment of the trap may be energized at 500 kHz so that cells emerging subsequently may be trapped there together with the beads that were mixed with them. At appropriate time intervals, the 3rd, then the 2nd, and finally the 1st trap may be similarly energized at 500-kHz. After completion of this process 5-different cell fractions may have been isolated and trapped, each containing cells that emerged from MAG-DEP-FFF separation between different time limits together with beads that were mixed with them. Although here described with respect to five segments, those having skill in the art recognize that any number of segments may be used.

[0068] Based on the inventors' knowledge of DEP-FFF and predictions about MAG-DEP-FFF, cells combining the smallest sizes, most uncomplicated surface morphologies and lowest concentrations of magnetically-labeled surface markers may emerge early and be trapped in segment 5. Conversely, cells combining large size, complex surface

morphology and high concentrations of surface markers may emerge last and be trapped in segment 1.

Histological Analysis of Cell Isolates

- 5 [0069] Optionally, the cells trapped in the different segments of the isolation and analysis stage may be treated with antibodies or stains by injecting these through the reagent port provided for this purpose. So long as the histological reagents do not affect cell viability, the cells may be held in place by positive DEP during perfusion and treatment. Several staining steps can be used and excess reagents or antibodies washed away, as needed. Glass and/or clear PDMS may be used for constructing the separation chambers. Therefore, after staining, cells isolated in the five segments may be compared and contrasted *in situ* by optical and/or fluorescence microscopy by a pathologist. If desired, additional reagents for the next step of cell analysis may be added at this point.

Focusing/Concentration

- 15 [0070] Having trapped cells and beads on the five segments of the isolator stage, optionally examined them with histological stains, and perfused them with the reagents needed for the next step in analysis, the cells may be focused to form a dense mixture with the beads. To accomplish this, the spiral electrodes in all five segments of the isolation stage may be energized with a four-phase field swept in frequency from 10 kHz to 200 kHz to provide a twDEP force directed towards the center of each of the five spirals. Because of the established dielectric properties of mammalian cells and the customized dielectric properties of the beads, this may sweep cells and beads of all types towards the center of the spiral on which they were originally trapped. It is believed that this process should take no more than 1 minute and should result in a dense conglomeration of cells and beads at the center of each spiral. In this way, each isolated fraction may be concentrated to a density of ~10⁹ cells/ml together with ~10¹⁰ beads/ml suspended in the reagent mixture that was perfused prior to focussing.

25 *Cell Bursting*

- 30 [0071] Once the cells and beads are concentrated, electro-mediated lysis of the cells can occur. This may be achieved by applying a strong AC voltage to the spiral electrode (e.g. 15 V peak to peak). Those having skill in the art recognize, however, that any other voltage suitable to cause bursting may be used.

Molecular Analysis

- 35 [0072] The liberation of intracellular components following cell lysis may allow their reaction with the perfused reagents and their interaction with the surfaces of beads (if present). Based on experiments reported in the literature for the hybridization of rare mRNA's in concentrated cell lysates with probes carried on beads, these reactions occur very rapidly, typically within a few minutes.

Detection

- 40 [0073] After an incubation time of 15 minutes, the target mRNA's should have hybridized with complimentary probes on beads. The spiral electrode segments may be energized with a 500 kHz non-travelling field to trap the beads at this point. Cell debris is not attracted by positive DEP and may be washed away from the beads. Indeed, relatively harsh reagents can be added to clean up the beads at this point providing those do not degrade the mRNA's bound to different bead types or damage the beads. After washing the beads free of debris and unhybridized molecules, the beads may be perfused with secondary fluorescent probes for target mRNA sequences. In this way, target sequences on the bead surfaces may be fluorescently labeled. Following additional washing steps to remove unbound secondary labels, the spiral electrodes may be energized with a 10 kHz signal to release the beads. At this point, eluate flow may be commenced through one spiral segment after another and the beads may be examined as they pass through the proximal impedance sensors.

- 45 [0074] Simultaneous fluorescence analysis may be used to quantify the amount of mRNA secondary label bound to each bead, and the AC impedance characteristics may be used to identify each bead/probe-combination (and hence index the mixed assays). This process should take about 15 minutes.

55 *Total Analysis Time*

- [0075] If all steps shown above were to be undertaken, the entire analysis from start to finish may take about 2 hours. This would include prefiltering cells from a starting mixture with a detection limit that should approach 1 cancer cell per

10⁶ normal cells; isolating tumor cells based on their dielectric properties and, optionally, surface immunomagnetic markers; histological analysis of the cells in comparison with other isolates; and molecular analysis for up to 10 different mRNA's.

5 [0076] Alternatively, if immunomagnetic markers and histology steps were omitted, the cell sorting, isolation, and molecular analysis would take about 45 minutes from start to finish.

Example 2 - Fabrication Issues

Fabrication of Electrodes

10 [0077] Electrode arrays may be fabricated using standard microphotolithographic techniques. Briefly, one may start with a clean glass substrate coated with 70Å titanium and 1000Å gold. Coating to NNN-S-450 specification may be done either commercially by Thin Film Technology, Inc., and guaranteed to be of uniform deposition, pinhole-free quality and able to withstand 10,000 psi lifting force, or using sputtering. The resulting gold blanks (up to 125 mm x 125 mm 15 in size) may be spin coated with Shipley photoresist which is exposed to UV light through a mask using a mask aligner (AB Manufacturing, San Jose). The resulting pattern is developed and inspected and the gold and titanium layers are then etched in two steps with KI/I₂ and hot HCl, respectively. Masks are designed by an IC CAD layout package (Design Workshop). Masks are either made commercially by the e-beam method (masks up to 6" x 6" and features down to <1µm) or else produced by photographically reducing a 10 x version of the mask printed on, for instance, a Hewlett-Packard DesignJet 2500CP printer at 600 dpi (final mask size up to 4.8" x 4.8" and features down to 4µ m).

20 [0078] To prevent cell sticking, electrodes may be silanized to produce a hydrophobic coating or else coated with TEFLON. Silanization is routinely accomplished with SigmaCote. TEFLON coating is accomplished by solvent deposition from a fluomcarbon carrier and subsequent baking onto silanized electrodes or by sputtering (in collaboration with the Stanford Microfabrication Laboratory).

Device structural fabrication

25 [0079] The glass substrate of the electrode array constitutes the lower wall of the device. Two approaches may be taken to construct device tops. In the first, the top wall consists of 4 mm glass into which holes are drilled for inlet and outlet port connections using a triple-tipped diamond drill. PEEK or TEFLON tubes are glued into the holes and cut off flush on what may become the inside surface of the device to form fluid interconnects. The two facing walls of the device are either sealed along their long edges with UV-curing epoxy glue, held in place by multiple small plastic clamps, or clamped by a single metal frame machined for the purpose. Fluid flow paths inside the device are defined in this construction method by a gasket of between 50 and 400 µm thickness, as required, having a slot cut wherever fluid flow is desired. The inventors have successfully used gaskets of PTFE, Gore-Tex, RTV and PDMS polymers. This method is adequate for simple flow paths but for the more complex flow paths in the integrated microfluidic component required for the multiple-segment spiral isolation and impedance sensing stage, a method using injection molded seals may be used. Seals may be made for this purpose in a separate mold and then sandwiched between a plain top and bottom as described above or the top of the device may be machined from Lucite and have seals injection molded directly into it. In this case the seals are made to extend above the surface of the top plate by a distance equal to the desired channel thickness. Simply pressing the device top plate against the device bottom then forms the required flow path and this allows for easy disassembly and cleaning without damaging a gasket. The molding material used to form the seals is PDMS, a resilient polymer that is durable, biologically inert, sufficiently compressible to form a good seal against fluids even with limited compression force, and transparent. In order to realize complex seal patterns, the inventors use a small Sherline CNC milling machine that operates directly from a CAD layout. In this way, flow paths that are mathematically defined can be cut directly into device top blanks under computer control. This allows well-defined, smooth fluidic pathways to be fabricated quickly and reproduced easily.

Fluid flow control

50 [0080] Fluid flow may be controlled by digital syringe pumps (KD scientific, Boston, Mass) each capable of holding two-syringes of different-barrel-sizes. The inventors have found that the useful-flow-rate from these pumps (i.e., for which there is an effective absence of pulsations due to stepper motor action) extends over 7 decades from 0.01 µl/min to 70.57 ml/min. For the fully integrated system as many as four pumps may be needed to allow automated sample control in the DEP prefilter, DEP-MAP-FFF stage and isolator. The pumps can be daisy-chained for convenient serial control by computer or manually controlled. Flow valves may be needed to control some waste and outlet lines. These can all be mounted off the fluidic device. Low dead volume valves from Lee may be used for these fluid control needs.

Conductivity measurements

[0081] Conductivity measurements of suspending medium solutions may be made with a Cole-Parmer 19101-00 electronic conductivity meter using either a flow-through or dip electrode cell with platinum black coated platinum electrodes.

Microscopy

[0082] Devices under test may be mounted on the stage of a Zeiss Axiovert S-100 inverted microscope (magnification X5 - X600) equipped with video recording and image analysis capabilities. This allows direct observation of any section of the transparent-walled devices and permits manual or automated visualization of cells. The microscope is equipped with epifluorescence and a sensitive three color CCD camera that is used for fluorescence microscopy. By quantifying the signal with software, fluorescence of molecular probes may be accomplished. For detection of molecular probe fluorescence signals, the inventors have an Oriel MS257 high sensitivity fiber optic tuneable dye laser spectrometer system and a Zeiss Axiovert 405M inverted microscope equipped with a Photometrics CH210 liquid nitrogen cooled photon-counting camera.

Electrical signals

[0083] Electrical fields for DEP/FFF and DEP trapping may be provided from 2 Hewlett-Packard 33120 signal generators (up to 15 V peak-peak, frequencies up to 50 MHz) with FM and AM sweeping capabilities. For twDEP focusing on the spiral electrode, four sine signals in quadrature are required and a digitally synthesized source based on a quadrature-phase numerically controlled oscillator chip may be used. This may be interfaced to a computer to provide quadrature signals up to 12 MHz and up to 12 V peak-peak with modulation characteristics that can be software controlled. Signals may be monitored with a Tektronix 200 MHz digital oscilloscope.

Magnetic fields

[0084] An important task in developing the DEP-MAP-FFF method is designing magnetic components to provide field distributions that achieve an appropriate distribution of $\bar{B} \nabla \cdot \bar{B}$ throughout the separation chamber. The design for the magnet system is shown in FIGS. 10 and 11. This arrangement of magnetic pole pieces may allow the field to be produced over the large area needed for a full sized DEP-MAP-FFF separator. Parallel SmCo or NdFB permanent magnets (e.g., 0.5 Tesla) may be used to provide fields closer to 1 Tesla. The field enhancement may be accomplished by exploiting boundary conditions on \bar{B} and \bar{H} at the iron surface. The enhancement is controlled by the shape of the Fe component and, in particular, by the size of the effective pole face. Field inhomogeneity may be controlled by the sintered iron particles underneath the DEP-MAP-FFF separation channel. In fact, principles used for creating MAP forces in the DEP-MAP-FFF separator are the same as used in existing MACS separators. However, the iron field enhancer and shapers may rely upon a well-defined microgeometry rather than the random geometries used in present day MACS separators. It should be borne in mind that the MAP forces needed to control the height of cells in a flow stream are about an order of magnitude less than those needed to trap cells in a column against hydrodynamic forces. For this reason the inventors believe that SmCo or NdFB magnets may be adequate.

[0085] Magnetic simulations may be undertaken while magnets are being built and tested using directional Hall probes to ascertain the field strengths and spatial inhomogeneity properties. In this way, design, simulation, construction, testing and refinement steps may go hand-in-hand to produce magnets suitable for the MAP requirements of this project.

Computer simulations

[0086] The distribution of the electrical and magnetic fields within the fluid between the chamber walls determines the DEP and MAP forces experienced by cells. Although the inventors' early electric field calculations were performed by the charge density method, implemented by FORTRAN, more recently the inventors have used the ANSYS multi-physics finite element analysis package to compute field distributions and have used the post-processing capabilities of MATLAB to derive the corresponding DEP force distributions.

[0087] DEP electrode geometries known in the art may be used. To achieve optimal $\bar{B} \nabla \cdot \bar{B}$ distributions for DEP-MAP force balance, however, one may need to use the ANSYS package to do simulations as a function of the size, shape and placement of the magnets, the iron field concentrator, and the sintered iron components. The ANSYS package allows simultaneous electrical and magnetic computations so that it is ideal for modeling the behavior of the DEP-MAP force balance properties of various geometries.

[0088] Finally, the ANSYS package also allows modeling of hydrodynamic characteristics of flow channels and the inventors plan to model the behavior of the fluid and cells as they pass though the integrated device, particularly in the fluid inlet and egress regions. This may be important in the interface regions between stages of the system to ensure the design allows efficient sample transport without "dead" spaces in which cells may settle.

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DEP trapping

[0089] Where needed, a 500 kHz field at 5 V p-p may be used to trap cells by DEP. This frequency is sufficiently high to penetrate the cell membranes efficiently without causing damage and induces a strong DEP body force on the cells, trapping them efficiently against fluid flow. DEP trapping may be used in four ways within the integrated system: (1) for cells being concentrated in the second segment of the prefilter following elution of normal cells and for small samples injected directly before the DEP-MAP-FFF stage; (2) for cell subpopulations that are isolated in the spiral electrode segments after elution from the DEP-MAP-FFF stage; and, (3) for holding cells in place during reagent perfusion at several steps in processing; (4) for holding beads in place for reagent perfusion following cell lysis and hybridization steps.

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DEP-MAP-FFF separation

[0090] Based on the inventors' experience with DEP-FFF, up to 2×10^5 cells can be analyzed without cell concentration becoming so large as to cause perturbing cell-cell interactions in the size of DEP-MAP-FFF fractionator chosen here. For samples expected to have a high concentration of suspect cells, such as dispersed cells from biopsies of suspected tumors or fine needle aspiration biopsies, 2×10^5 cells are sufficient to ensure that tumor cells, if present, may be sufficient for molecular analysis. In such cases, up to 20 μL of cell suspension may be injected via the preconcentrated sample loading port. For samples in which the concentration of suspect cells is expected to be so low that there is unlikely to be sufficient suspect cells in a 2×10^5 cell sample, prefiltering may be necessary. Samples such as peripheral blood mononuclear cells or dispersed lymph node cell populations fall into this category.

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[0091] Following injection of a 20 μL sample or prefiltering, as appropriate, the secondary trapping electrode may be energized at 250 kHz frequency and 5 V p-p. All cell types may be trapped from the flow stream by DEP on the electrode in the entrance region of the DEP-MAP-FFF separator stage. Sample injection into the DEP-MAP-FFF stage may now occur with an appropriate DEP levitation signal applied. After cells have been given time to reach equilibrium heights (2-5 minutes) under the influence of DEP, MAP and gravitational forces, carrier medium flow may be started from a digital syringe pump (KD scientific, Boston, Mass). The first cell subpopulations should begin emerging from the DEP-FFF fractionator approximately 2-5 minutes after the initiation of fluid flow. Frequencies from 10 kHz to 500 kHz, voltages from 0.5 V p-p to 3 V p-p, and carrier fluid conductivities from 5-1000 mS/m may be used.

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Cell tracking

[0092] Cell fractionation, isolation, concentration and bursting may be investigated in the integrated devices. Cultured breast tumor cells may be mixed with PBMCs to provide a well-characterized and reproducible model system for investigating the performance and optimal operating conditions for the component parts of the integrated system. To assist in tracking the cell subpopulations, one may initially prelabel the breast cancer cells to facilitate tracking. This may be done in two ways. Initially, cells may be incubation for 10 mins in 25 $\mu\text{g}/\text{ml}$ BCECF-AM (Molecular Probes), a fluorescein probe that is irreversibly accumulated by cells through the action of nonspecific esterases. BCECF is only accumulated by viable cells and simultaneously acts as a viability indicator. In experiments, such labeling allowed convenient tracking of tumor cells which appeared as brilliant spheres against a dark field of unlabelled cells, allowing even a single tumor cell within a very large unlabelled population ($> 10^5$ cells) to be instantly identified. This tracking technique may be used to study the cells by fluorescent microscopy while they are undergoing separation and manipulations in the device.

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[0093] Secondly, FTTC-conjugated human epithelial antigen (HEA) antibody may be used to prelabel breast cancer cells prior to adding them to PBMNC mixtures. The fluorescence of this labeling procedure is much weaker than BCECF, however cells emerging from the separator stages can be passed directly into a flow cytometer and definitively identified as being-of epithelial origin-by-this method.

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[0094] *Cell and cell culture:* For model studies, one may use MDA-MB-435, MDA-MB-453, MDA-MB-236, and MDA-MB-468 human breast cancer lines originally established by Cailleau et al. as well as MCF-7 originally from the Michigan Cancer Foundation. These have formed the basis for investigations into many aspects of tumorigenesis and metastasis, are well characterized, and are available from ATCC to other researchers for follow-up studies. MDA-MB-453 shows a 64-fold enhancement in mRNA level of HER2/neu compared with MDA-MB-231 and a comparable increase in cell surface concentration of the corresponding protein and is therefore suitable for both immunological and

mRNA assays. Tumor cells are cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM glutamine and 20 mM HEPES buffer in 25-cm² vented culture flasks (Costar) at 37°C under a 5% CO₂/95% air atmosphere. Cultures are free of, and are periodically checked by radionucleic acid hybridization assay (Gen-Probe, Inc.) for, mycoplasma. Cells are harvested from 50-70% confluent cultures by brief exposure to 0.25% trypsin-0.02% EDTA solution. Viability is determined by trypan blue dye exclusion.

[0095] Samples for DEP fractionation and manipulation may be prepared by suspending cells in sucrose/dextrose solution to yield suspensions having a specified conductivity of between 10 and 1000 mS/m and physiological osmolarity (300 mOs/kg). If necessary, conductivity is adjusted with additional culture medium.

10 *Immunological detection*

[0096] Cell samples can be incubated with antibodies for markers prior to loading into the separation stages, while at the interface between the prefilter and DEP-MAP-FFF fractionator stages, and after trapping in the spiral electrode isolator stage prior to concentration. A series of DEP levitation/trapping cycles can be applied to "stir" the antibody/cell mixture at each of these steps. Following labeling, cells may be trapped by positive DEP and washed free of antibodies by perfusing them with rinsing reagents as many times as needed. Fluorescently, magnetically or enzymically labeled antibodies can be used. Fluorescence microscopy can be used to detect fluorescence of the antibodies or of their catalytic by-products. Immunomagnetic labels may modify the DEP-MAP-FFF properties of cell types in accordance with their surface marker concentrations. One may use antibodies for human epithelial antigen (HEA) because this is a useful marker for identifying epithelial cells in blood and lymph node cell dispersions, and EGF receptor antibody since this is a relevant prognostic marker for breast cancer. Clearly, these examples are merely exemplary of the more general applicability of the technology and surface markers relevant to any different application could be used instead.

25 *twDEP focusing/concentration of cells*

[0097] The twDEP properties of blood and cultured breast cancer cell lines are known in the art. A traveling wave field applied to the spiral electrode array at a frequency that both levitates and translates a cell subpopulation may allow it to be focused at the center of the spiral. A swept frequency may be applied to ensure that all cell and bead types on each spiral isolation segment may be swept to the center to form a highly concentrated mixture. Traveling waves in the frequency range 10 kHz to 500 kHz, voltages from 0.5 V p-p to 5 V p-p, and carrier fluid conductivities from 5-1000 mS/m may be used.

30 *Computer control*

35 [0098] In one embodiment, the pumps and signal generators used to operate the system are all computer controllable. Image processing may use a dual-Pentium II PCI/EISA mother board. The image grabber may include a real-time image processor (Image Series 640+Neighborhood Processor with on-board 4 MB memory, Matrox Electronic Systems Ltd., Dorval, Canada) that is used to acquire images and to accelerate image operations. Appropriate software known in the art performs real-time process control of the serial and HPIB devices (pumps, valves, signal sources, digital camera) used to operate the system and a real-time imaging library (MIL-32 3.10, Matrox Electronic Systems Ltd., Dorval, Canada) used in conjunction with Labview software may be exploited for system control and fluorescence detection.

45 *Bursting of cells*

[0099] Following the trapping of cell fractions on the spiral electrode segments and their concentration by twDEP, the voltage and frequency applied to the spiral electrode may be changed to burst the target cells. A further level of cell discrimination is possible at this stage because targeted bursting can be done on cell mixtures if desired. Breast cancer cells are typically in the 10-12 µm diameter range and have specific membrane capacitances of ~20 mF/m². These parameters in conjunction with the suspending medium conductivity define the optimum bursting conditions. These may be examined for target cultured breast cancer and human specimen cells for carrier fluid conductivities from 5-1000 mS/m. Optimum field conditions for rapidly bursting all cells on the spiral electrode may also be determined. Voltages from 10 V peak-peak to 20V peak-peak and frequencies from 10 kHz to 100 kHz may be used, including swept frequencies.

55 [0100] While the present disclosure may be adaptable to various modifications and alternative forms, specific embodiments have been shown by way of example and described herein. However, it should be understood that the present disclosure is not intended to be limited to the particular forms disclosed. Rather, it is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the disclosure as defined by the appended claims.

Moreover, the different aspects of the disclosed apparatus and methods may be utilized in various combinations and/or independently. Thus the invention is not limited to only those combinations shown herein, but rather may include other combinations.

5 **Example 3 - Programmable Fluidic Processor**

[0101] In one embodiment of the present invention, a programmable fluidic processor (PFP) may be coupled to the array isolator that may be coupled to the electrode array isolator that is used to trap cells after they exit from the field-flow fractionation separator. Various embodiments of the PFP are discussed in pending U.S. Application No. 09/249,955, which has been previously incorporated herein by reference.

[0102] As previously indicated, the array isolator may consist of a plurality of spiral traps. The PFP may be coupled to the spiral traps by a variety of means known in the art. For example, the PFP may be coupled to the spiral traps by means of a channel, or the PFP may be integral with the spiral traps. There may be one or more PFPs. Each spiral trap may have its own PFP, or multiple spiral traps may be connected to a single PFP.

15 Once the cells have been trapped on the spiral traps, they may be moved to the PFP for further analysis. Once the cells have been transferred, the PFP may be used to programmably manipulate the cells in a variety of ways. FIG. 12 shows one embodiment of the present invention that includes a PFP. As shown in FIG. 12, a single PFP may be connected to each of the spiral traps.

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5 **Claims**

10 1. A fluidic device for the analysis of cells, the device comprising:

(a) a dielectrophoretic field-flow fractionation separator comprising a fluidic channel and an array of micro-electrodes configured to discriminate cells by balancing a dielectrophoretic force with a gravitational force to position different cell types to different heights above the floor of the fluidic channel within a velocity profile in the separator so that different cell types are carried through the fluidic channel at different velocities to become separated, the separated different cell types emerging from a far end of the channel; **characterized by**
(b) a multi-element electrode array isolator comprising a plurality of electrode elements coupled to the separator and configured so that each electrode array element traps a separate fraction of cells as a function of the cells' time of emergence from the separator.

20 2. The device of claim 1, wherein the electrode elements are spiral electrode elements.

25 3. The device of claim 1, the device further comprising

- a programmable fluidic processor coupled to the electrode array isolator.

4. The device of claim 2 or 3, the device further comprising

- a dielectrophoretic prefilter coupled to the separator, the prefilter comprising one or more trapping electrodes configured to trap at least a portion of the cells with a dielectrophoretic force.

30 5. The device of claim 1, wherein the separator further comprises a magnet configured to displace with a magneto-phoretic force the cells to positions within the velocity profile in the separator.

35 6. The device of claim 5, wherein the magnet are several flat magnets of SmCo or NdFeB materials placed at a parallel configuration in an opposing pole orientation.

7. The device of claim 1, wherein each of the plurality of spiral electrode elements is configured to be energized by a signal of a single frequency, but wherein the phase of the signal is different for each of the plurality of electrode elements.

40 8. The device of claim 7, the device further comprising four electrode elements, and wherein the phases of the signal are 0°, 90°, 180°, 270°.

45 9. The device of claim 4, the device further comprising

- a reagent port configured to allow for the injection of reagents onto the cells trapped on the spiral electrode elements.

50 10. A method for cell isolation and analysis, the method comprising the steps of:

- introducing cells-into-the-dielectrophoretic field-flow fractionation separator-as-defined-in claim-1; -
discriminating the cells in the separator, the step of discriminating comprising the step of balancing a dielectrophoretic force with a gravitational force to displace the cells to positions within a velocity profile in the separator; and

55 trapping at least a portion of the cells emerging from the separator with the multi-element electrode array isolator comprising a plurality of spiral electrode elements, as defined in claim 1, as a function of the cells' time of emergence from the separator.

11. The method of claim 10, the method further comprising the step of manipulating the cells with a programmable fluidic processor coupled to the electrode array isolator.
12. The method of claim 10, the method further comprising the step of lysing the cells trapped by the multi-element electrode array isolator, in particular wherein the step of lysing comprises using AC electrical fields.
13. The method of claim 10, wherein at least a portion of the cells is initially coupled to the surface of a carrier bead.
14. The method of claim 10 or 11, the method further comprising the steps of
 - 10 introducing the cells into the dielectrophoretic prefilter as defined in claim 3, thereby trapping at least a portion of the cells with a dielectrophoretic force, and directing the cells trapped from the prefilter into the separator coupled to the prefilter.
15. The method of claim 14, wherein a plurality of analysis beads is mixed with the cells after the cells emerge from the separator.
16. The method of claim 15, the method further comprising the step of concentrating the cells on the plurality of spiral electrode elements, the step of concentrating the cells comprising the step of energizing the plurality of spiral electrode elements with a multi-phase field, in particular wherein the multi-phase field comprises four phases, and comprises a frequency between 10 kHz to 200 kHz.
17. The method of claim 10, wherein the cells are incubated with magnetically labeled antibodies.

25 **Patentansprüche**

1. Fluidvorrichtung für die Analyse von Zellen, wobei die Vorrichtung aufweist:
 - 30 (a) einen dielektrophoretischen Feldströmungs-Fraktionierungsseparator, der einen Fluidkanal und eine Anordnung von Mikroelektroden aufweist, die dafür angeordnet sind, Zellen zu unterscheiden, indem eine dielektrophoretische Kraft durch eine Schwerkraft kompensiert wird, um unterschiedliche Zelltypen auf unterschiedlichen Höhen über dem Boden des Fluidkanals innerhalb eines Geschwindigkeitsprofil in dem Separator zu positionieren, so dass unterschiedliche Zelltypen durch den Fluidkanal bei unterschiedlichen Geschwindigkeiten zwecks Trennung transportiert werden, wobei die getrennten unterschiedlichen Zelltypen aus einem entfernten Ende des Kanals austreten; und
 - 35 (b) einen Mehrfachelement-Elektrodenanordnungsisolator, der mehrere Elektrodenelemente aufweist, die mit dem Separator verbunden sind und so konfiguriert sind, dass jedes Elektrodenanordnungselement eine getrennte Fraktion von Zellen als eine Funktion der Austrittszeit der Zellen aus dem Separator einfängt.
2. Vorrichtung nach Anspruch 1, wobei die Elektrodenelemente Spiralen-Elektrodenelemente sind.
3. Vorrichtung nach Anspruch 1, wobei die Vorrichtung ferner aufweist:
 - 45 - einen programmierbaren Fluidprozessor, der mit dem Elektrodenanordnungsisolator verbunden ist.
4. Vorrichtung nach Anspruch 2 oder 3, wobei die Vorrichtung ferner aufweist:
 - 50 - ein mit dem Separator verbundenes dielektrophoretisches Vorfilter, wobei das Vorfilter eine oder mehrere Einfangelektroden aufweist, die dafür konfiguriert sind, wenigstens einen Teil von den Zellen mit einer dielektrophoretischen Kraft einzufangen.
5. Vorrichtung nach Anspruch 2, wobei der Separator ferner einen Magneten aufweist, der dafür konfiguriert ist, mit einer magnetophoretischen Kraft die Zellen auf Positionen innerhalb des Geschwindigkeitsprofils in dem Separator zu verschieben.
6. Vorrichtung nach Anspruch 5, wobei der Magnet aus mehreren flachen Magneten aus SmCo oder NdFeB Mate-

rialien besteht, die in einer parallelen Konfiguration in einer gegenüberliegenden Polorientierung angeordnet sind.

7. Vorrichtung nach Anspruch 1, wobei jedes von den mehreren Spiral-Elektrodenelementen dafür konfiguriert ist, dass es durch ein Signal mit nur einer Frequenz erregt wird, wobei aber die Phase des Signals für jedes von den mehreren Elektrodenelementen unterschiedlich ist.

8. Vorrichtung nach Anspruch 7, wobei die Vorrichtung ferner vier Elektrodenelemente aufweist, und wobei die Phasen des Signals 0°, 90°, 180°, 270° sind.

10 9. Vorrichtung nach Anspruch 4, wobei die Vorrichtung ferner aufweist:

- einen Reagens-Anschluss, der dafür konfiguriert ist, die Injektion von Reagenzien auf die auf den Spiral-Elektrodenelementen eingefangen Zellen zu ermöglichen.

15 10. Verfahren zur Zellisolation und Analyse, wobei das Verfahren die Schritte aufweist:

Einführen von Zellen in den dielektrophoretischen Feldströmungs-Faktionierungsseparator gemäß Definition in Anspruch 1;

20 Unterscheiden der Zellen in dem Separator, wobei der Unterscheidungsschritt den Schritt der Kompensation einer dielektrophoretischen Kraft mit einer Schwerkraft umfasst, um die Zellen auf Positionen innerhalb eines Geschwindigkeitsprofils in dem Separator zu verschieben; und

25 Einfangen wenigstens eines Teils von aus dem Separator austretenden den Zellen mit dem Mehrfachelement-Elektrodenanordnungsisolator, der mehrere Spiral-Elektrodenelemente aufweist, gemäß Definition in Anspruch 1, als eine Funktion der Austrittszeit der Zellen aus dem Separator.

11. Verfahren nach Anspruch 10, wobei das Verfahren ferner den Schritt aufweist:

30 Manipulation der Zellen mit einem programmierbaren Fluidprozessor, der mit dem Elektrodenanordnungsisolator verbunden ist.

35 12. Verfahren nach Anspruch 10, wobei das Verfahren ferner den Schritt der Lösung der durch den Mehrfachelement-Elektrodenanordnungsisolator eingefangen Zellen aufweist, wobei insbesondere der Schritt der Lösung die Verwendung von elektrischen Wechselfeldern aufweist.

13. Verfahren nach Anspruch 10, wobei wenigstens ein Teil der Zellen zu Beginn mit der Oberfläche eines Trägerteilchens verbunden ist.

40 14. Verfahren nach Anspruch 10 oder 11, wobei das Verfahren ferner die Schritte aufweist:

45 Einführen der Zellen in das dielektrophoretisches Vorfilter gemäß Definition in Anspruch 2, um dadurch wenigstens einen Teil von den Zellen mit einer dielektrophoretischen Kraft einzufangen, und Weiterleiten der von dem Vorfilter eingefangen Zellen in einen mit dem Vorfilter verbundenen Separator.

15. Verfahren nach Anspruch 14, wobei mehrere Analyseteilchen mit den Zellen gemischt werden, nachdem die Zellen aus dem Separator austreten.

50 16. Verfahren nach Anspruch 15, wobei das Verfahren ferner den Schritt der Konzentration der Zellen auf den mehreren Spiral-Elektrodenelementen aufweist, der Schritt der Konzentration der Zellen den Schritt der Erregung der mehreren Spiral-Elektrodenelementen mit einem mehrphasigen Feld aufweist, wobei insbesondere das mehrphasige Feld vier-Phasen aufweist und eine Frequenz zwischen 10 kHz bis 200 kHz aufweist.

17. Verfahren nach Anspruch 10, wobei die Zellen mit magnetisch markierten Antikörpern inkubiert werden.

Revendications

1. Appareil basé sur le flux pour l'analyse de cellules, l'appareil comprenant :

- 5 (a) un séparateur par fractionnement par couplage flux-force diélectrophorétique comprenant un canal de flux et une rangée de micro-électrodes configurée pour différencier les cellules en équilibrant une force diélectrophorétique avec une force gravitationnelle pour positionner différents types cellulaires à différentes hauteurs au-dessus de la base du canal de flux à l'intérieur d'un profil de vitesse dans le séparateur de manière à ce que différents types cellulaires soient portés le long du canal de flux à différentes vitesses pour qu'ils se séparent, les différents types cellulaires séparés sortant d'une extrémité éloignée du canal ; **caractérisé par**
10 (b) un isolateur à rangée d'électrodes à plusieurs éléments, comprenant une pluralité d'éléments d'électrodes couplés avec le séparateur et configurés de manière à ce que chaque élément de la rangée d'électrodes piége une fraction séparée de cellules en fonction du temps de sortie du séparateur des cellules.

15 2. Appareil selon la revendication 1, dans lequel les éléments d'électrodes sont des éléments d'électrodes en spirale.

3. Appareil selon la revendication 1, l'appareil comprenant de plus

- 20 - un processeur fluidique programmable couplé à l'isolateur à rangée d'électrodes.

4. Appareil selon la revendication 2 ou 3, l'appareil comprenant de plus

- 25 - un pré-filtre diélectrophorétique couplé au séparateur, le pré-filtre comprenant une ou plusieurs électrodes de piégeage configurées pour piéger au moins une portion des cellules avec une force diélectrophorétique.

5. Appareil selon la revendication 1, dans lequel le séparateur comprend de plus un aimant configuré pour déplacer les cellules avec une force magnétophorétique à des positions à l'intérieur du profil de vitesse dans le séparateur.

30 6. Appareil selon la revendication 5, dans lequel l'aimant est constitué de plusieurs aimants plats de matériau SmCo ou NdFeB placés dans une configuration en parallèle dans une orientation à pôles opposés.

7. Appareil selon la revendication 1, dans lequel chacun de la pluralité d'éléments d'électrodes en spirale est configuré pour être stimulé par un signal à une seule fréquence, mais, dans lequel la phase du signal est différente pour chacun des différents éléments d'électrodes.

35 8. Appareil selon la revendication 7, l'appareil comprenant de plus quatre éléments d'électrodes, et dans lequel les phases du signal sont de 0°, 90°, 180°, 270°.

9. Appareil selon la revendication 4, l'appareil comprenant de plus

- 40 - un port de réactifs configuré pour permettre l'injection de réactifs sur les cellules piégées sur les éléments d'électrodes en spirale.

10. Procédé pour isoler et analyser des cellules, le procédé comprenant les étapes consistant à :

45 introduire des cellules dans le séparateur par fractionnement par couplage flux-force diélectrophorétique tel que défini dans la revendication 1 ;

distinguer des cellules dans le séparateur, l'étape de distinction comprenant l'étape d'équilibrage d'une force diélectrophorétique avec une force gravitationnelle pour déplacer les cellules jusqu'à des positions à l'intérieur d'un profil de vitesse dans le séparateur ; et

50 - piéger au moins une portion des cellules sortant du séparateur avec l'isolateur à rangée d'électrodes à plusieurs éléments comprenant une pluralité d'éléments d'électrodes en spirale, tel que défini dans la revendication 1, en fonction du temps de sortie du séparateur des cellules.

55 11. Procédé selon la revendication 10, le procédé comprenant de plus les étapes consistant à manipuler les cellules avec un processeur fluidique programmable couplé à l'isolateur à rangée d'électrodes.

12. Procédé selon la revendication 10, le procédé comprenant de plus l'étape de lyse des cellules piégées par l'iso-

lateur à rangée d'électrodes à plusieurs éléments, en particulier dans lequel l'étape de lyse comprend l'utilisation de champs électriques de courant alternatif.

5 13. Procédé selon la revendication 10, dans lequel au moins une portion des cellules est initialement couplée à la surface d'une bille porteuse.

10 14. Procédé selon la revendication 10 ou 11, le procédé comprenant de plus les étapes consistant à introduire les cellules dans le pré-filtre diélectrophorétique tel que défini dans la revendication 3, piégeant ainsi au moins une portion des cellules avec une force diélectrophorétique, et orienter les cellules piégées depuis le pré-filtre jusque dans le séparateur couplé au pré-filtre.

15 15. Procédé selon la revendication 14, dans lequel une pluralité de billes d'analyse sont mélangées avec les cellules après que les cellules sont sorties du séparateur.

20 16. Procédé selon la revendication 15, le procédé comprenant de plus l'étape de concentration des cellules dans une pluralité d'éléments d'électrodes en spirale, l'étape de concentration des cellules comprenant l'étape de stimulation de la pluralité d'éléments d'électrodes en spirale avec un champ à phases multiples, en particulier dans lequel le champ à phases multiples comprend quatre phases, et comprend une fréquence entre 10kHz et 200kHz.

25 17. Procédé selon la revendication 10, dans lequel les cellules sont incubées avec des anticorps marqués de manière magnétique.

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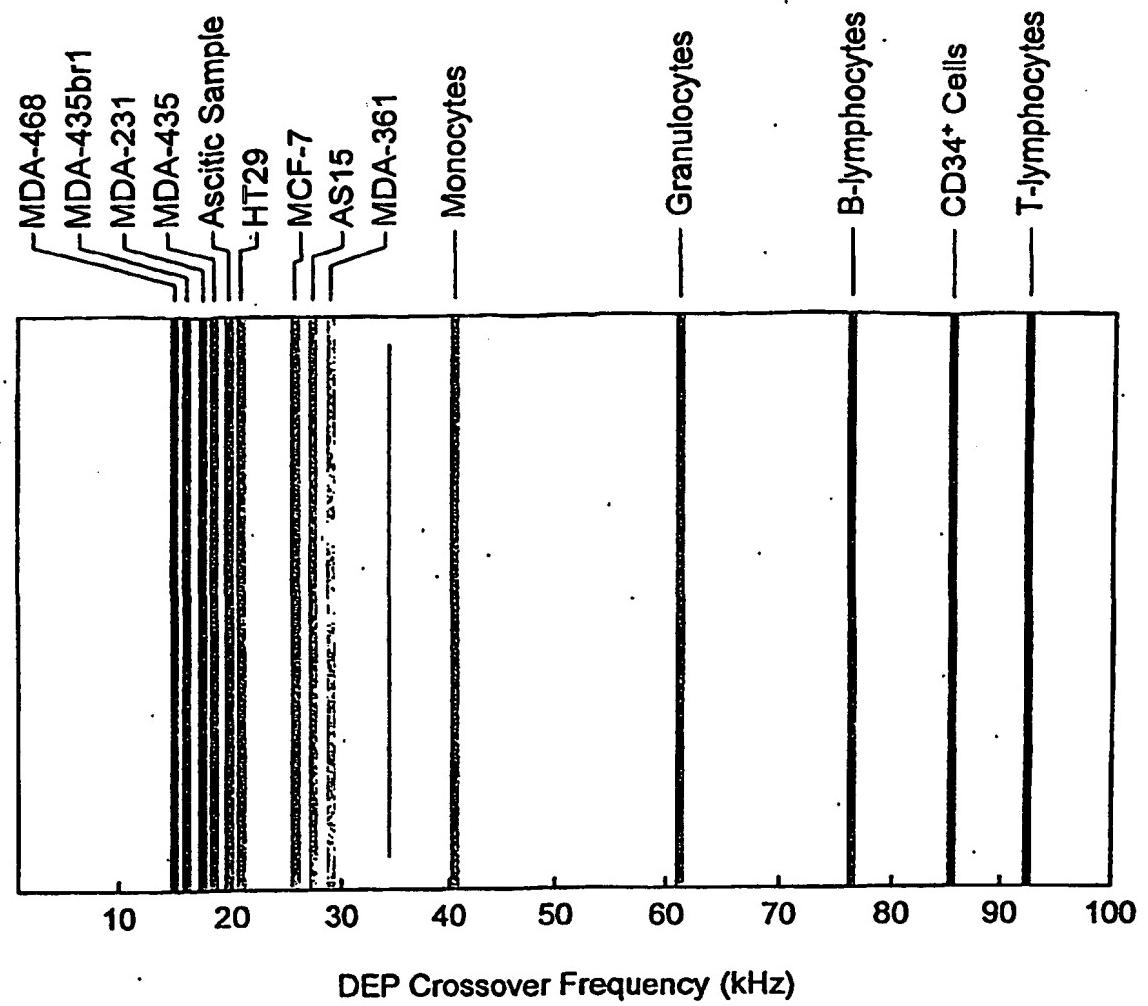


FIG. 1

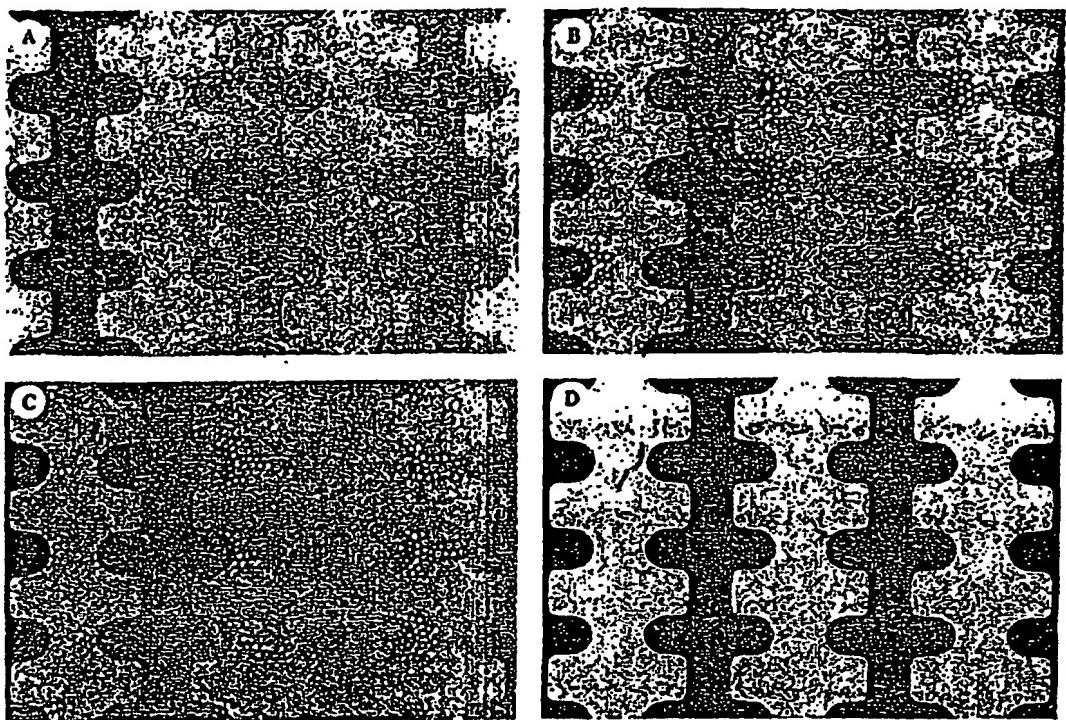


FIG. 2

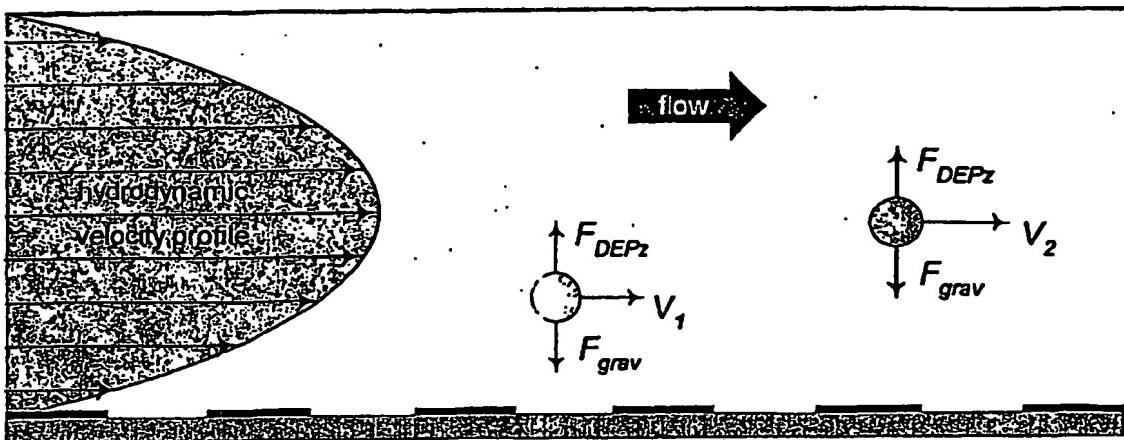


FIG. 3

Separation Summary

| Experiment Parameters | Cell Types | Ratio (before) | Purity (after) | Separation Time (min) |
|--|--|----------------|-------------------|-----------------------|
| 15-40 kHz, 5 min; 5 kHz, 7 min | MDA435 CD34+ Cells | 2 : 3 | 99% 99.2% | 12 |
| 5 kHz, 30 min | MDA435 Erythrocytes | 1 : 1 | 99.9% 99.9% | 30 |
| 15-35 kHz, 5 min; 5 kHz, 7 min) | MDA435 T-lymphocytes | 2 : 3 | 98% 92% | 12 |
| 20-50 kHz, 10 min; 5 kHz, 6 min | Monocytes T-lymphocytes | 1 : 1 | 98% 92% | 16 |
| 20-40 kHz, 10 min; 5 kHz, 6 min | Monocytes B-lymphocytes | 1 : 1 | 94% 92% | 16 |
| 40-50 kHz, 8 min; 5 kHz, 5 min | Granulocytes T-lymphocytes | 1 : 1 | 94% 87% | 13 |
| 30-35 kHz, 8 min; 5 kHz, 5 min | Monocytes Granulocytes | 1 : 1 | 97% 91% | 13 |
| 40-55 kHz, 5 min; 25-35 kHz, 5 min; 5 kHz, 5 min | T-lymphocytes Granulocytes Monocytes | 8 : 8 : 1 | 96% 91% 58% | 14 |
| 10 kHz, 25 min | Leukocytes Erythrocytes | 1 : 700 | 5% 99.99% | 25 |

FIG. 4

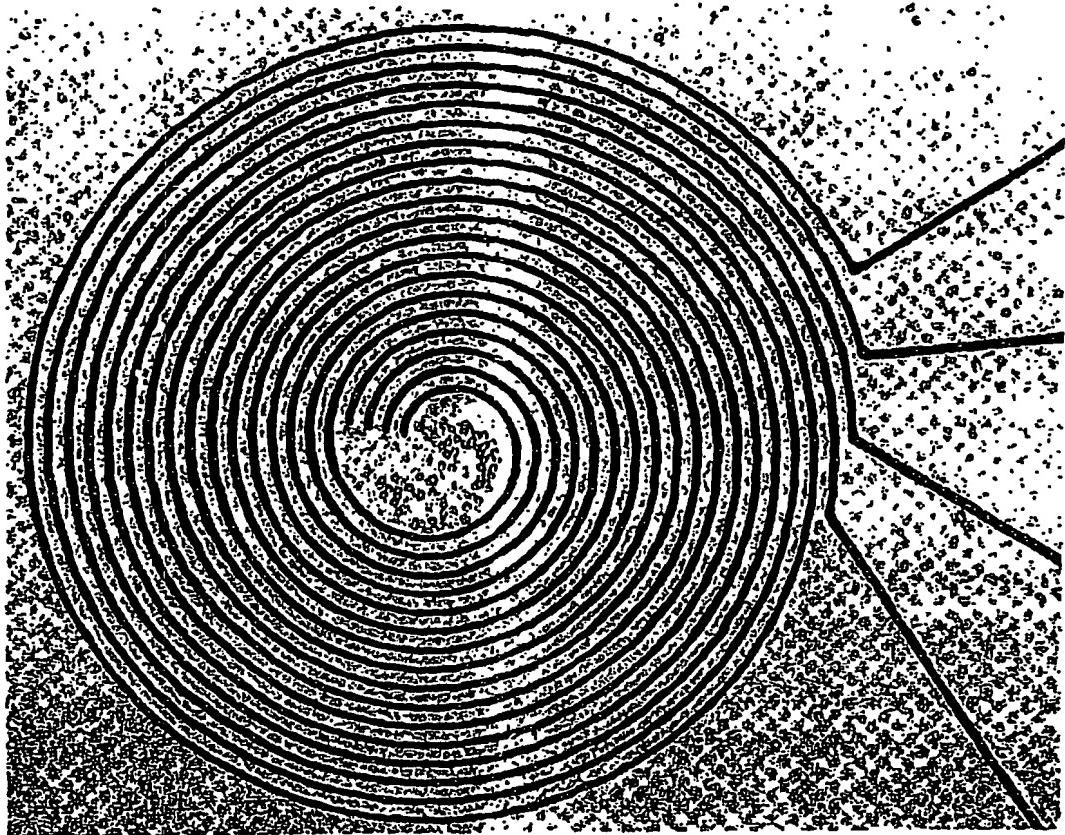


FIG. 5

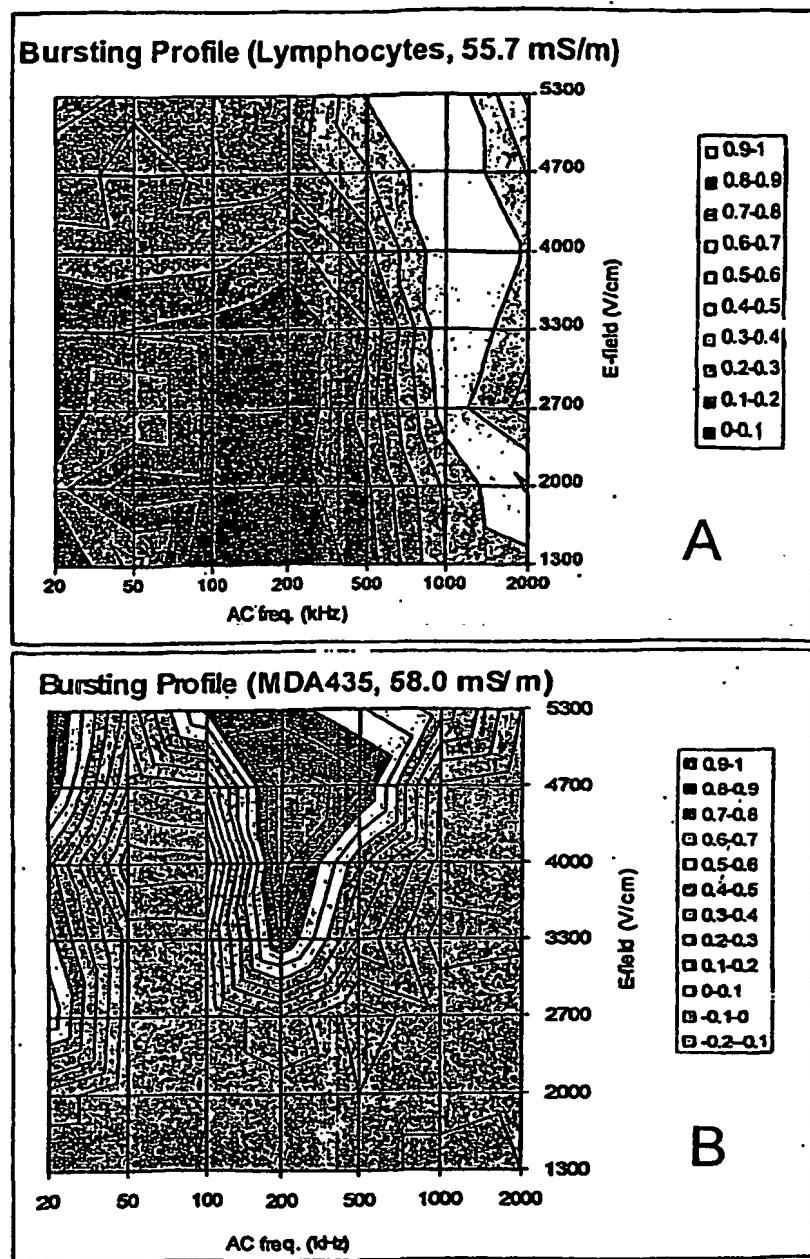


FIG. 6

Opposing 2200 G Fields
from SmCo Magnets

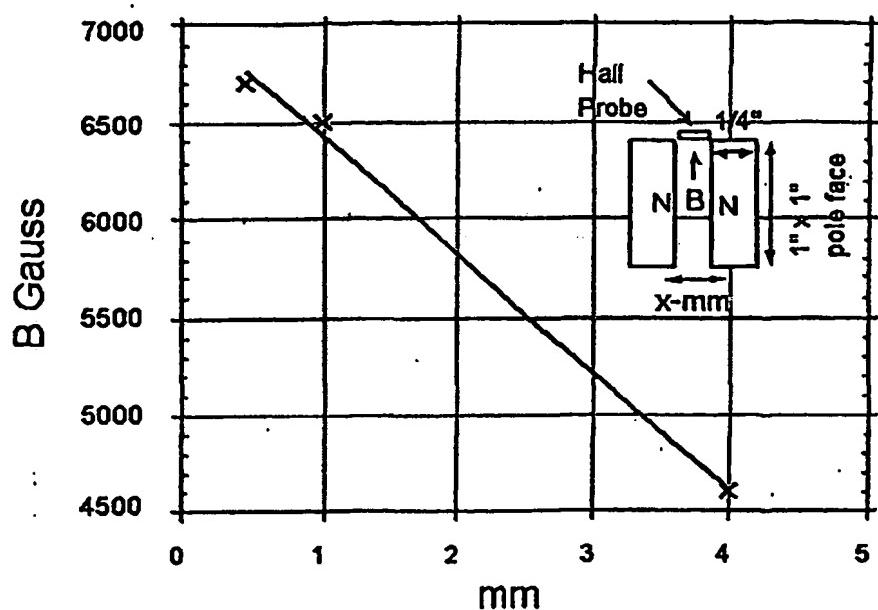


FIG. 7

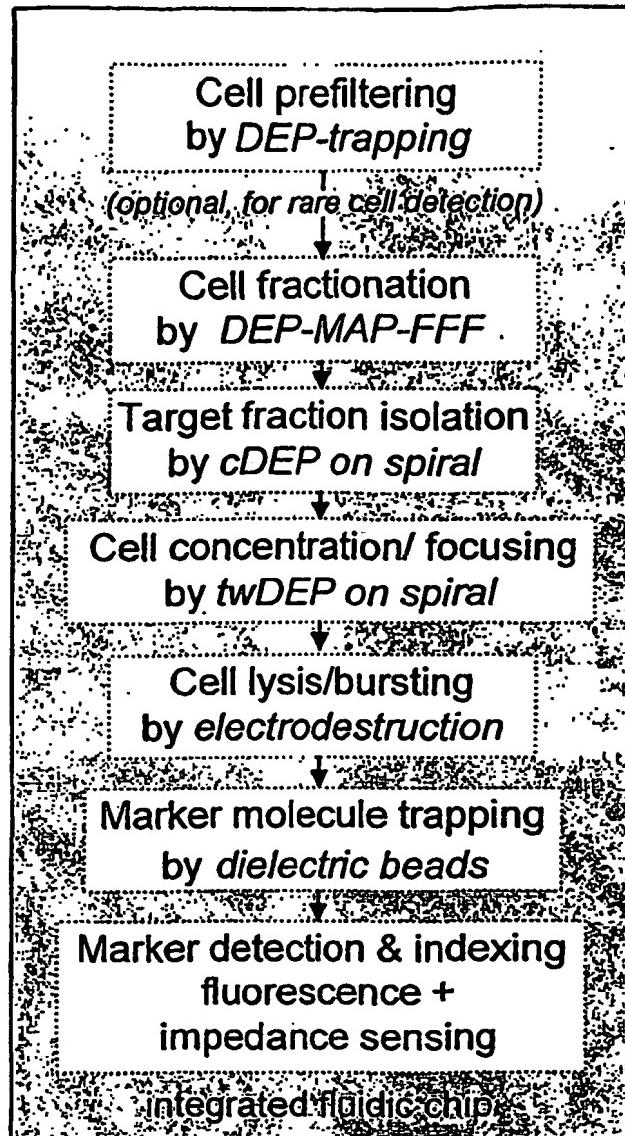


FIG. 8

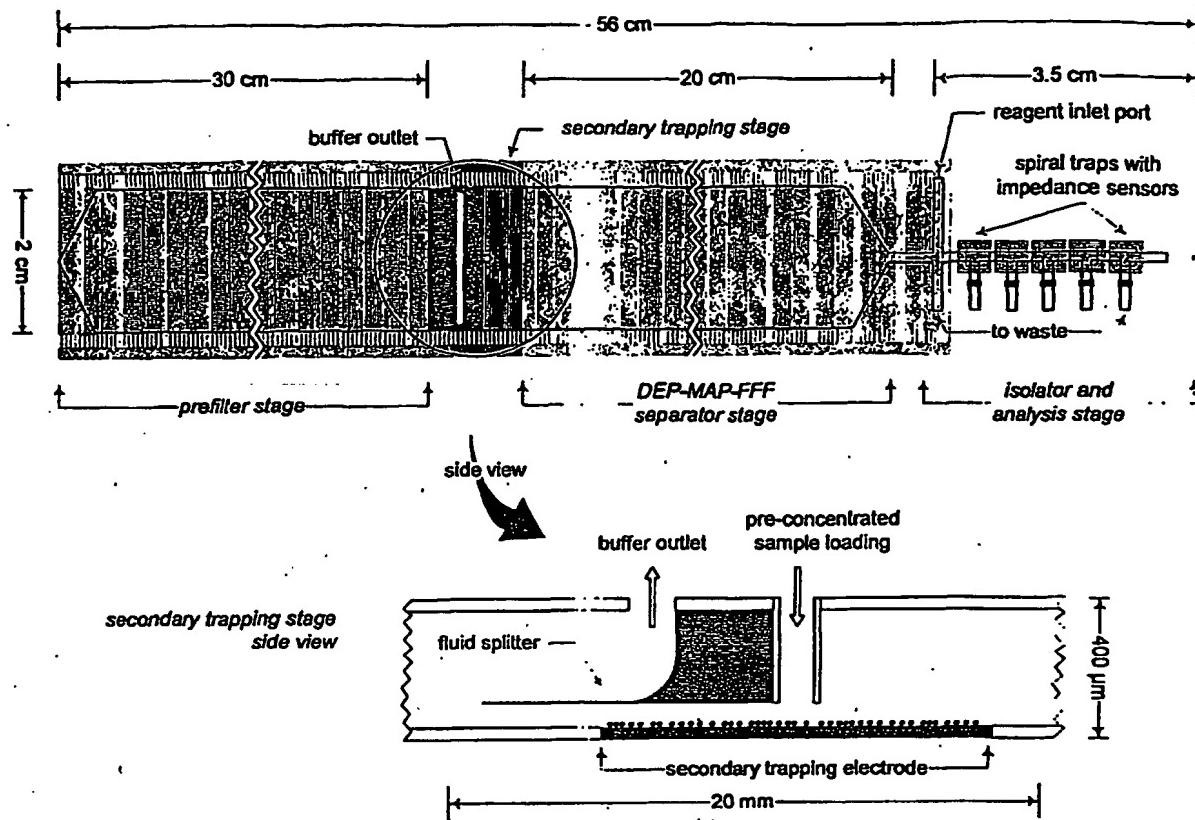


FIG. 9

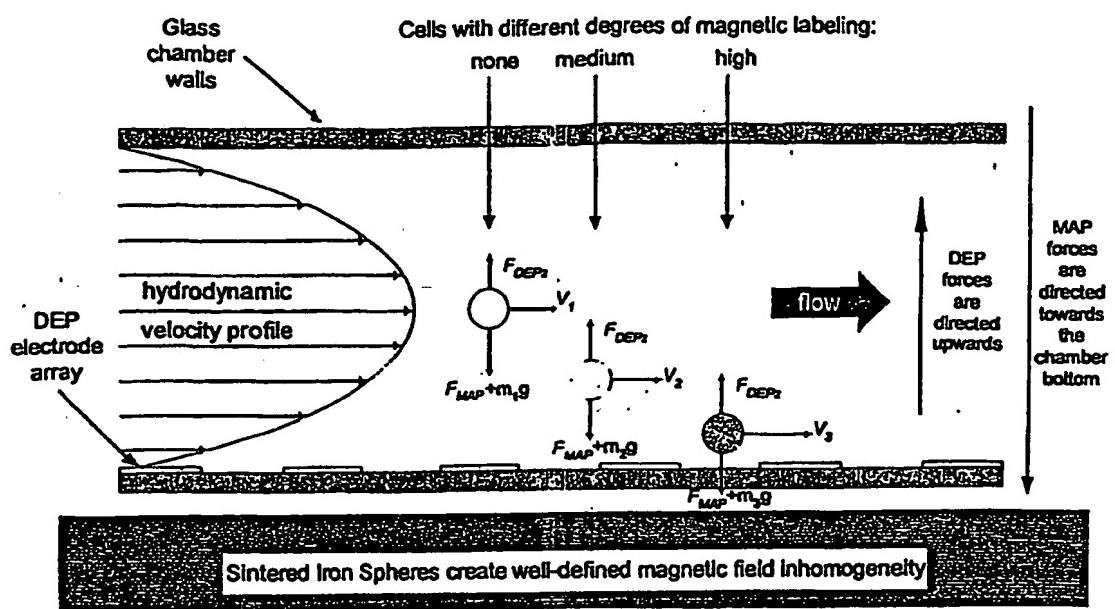


FIG. 10

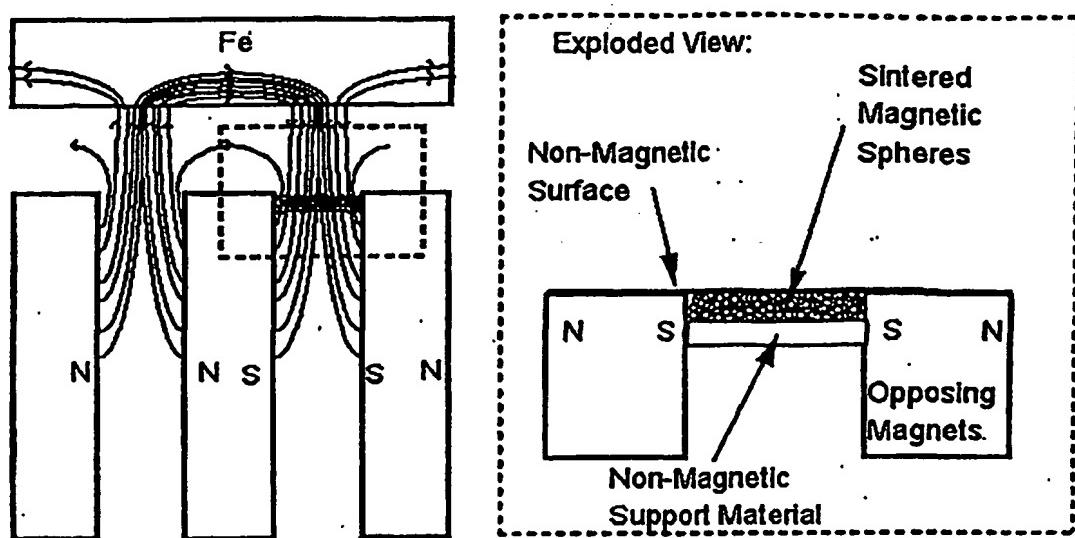
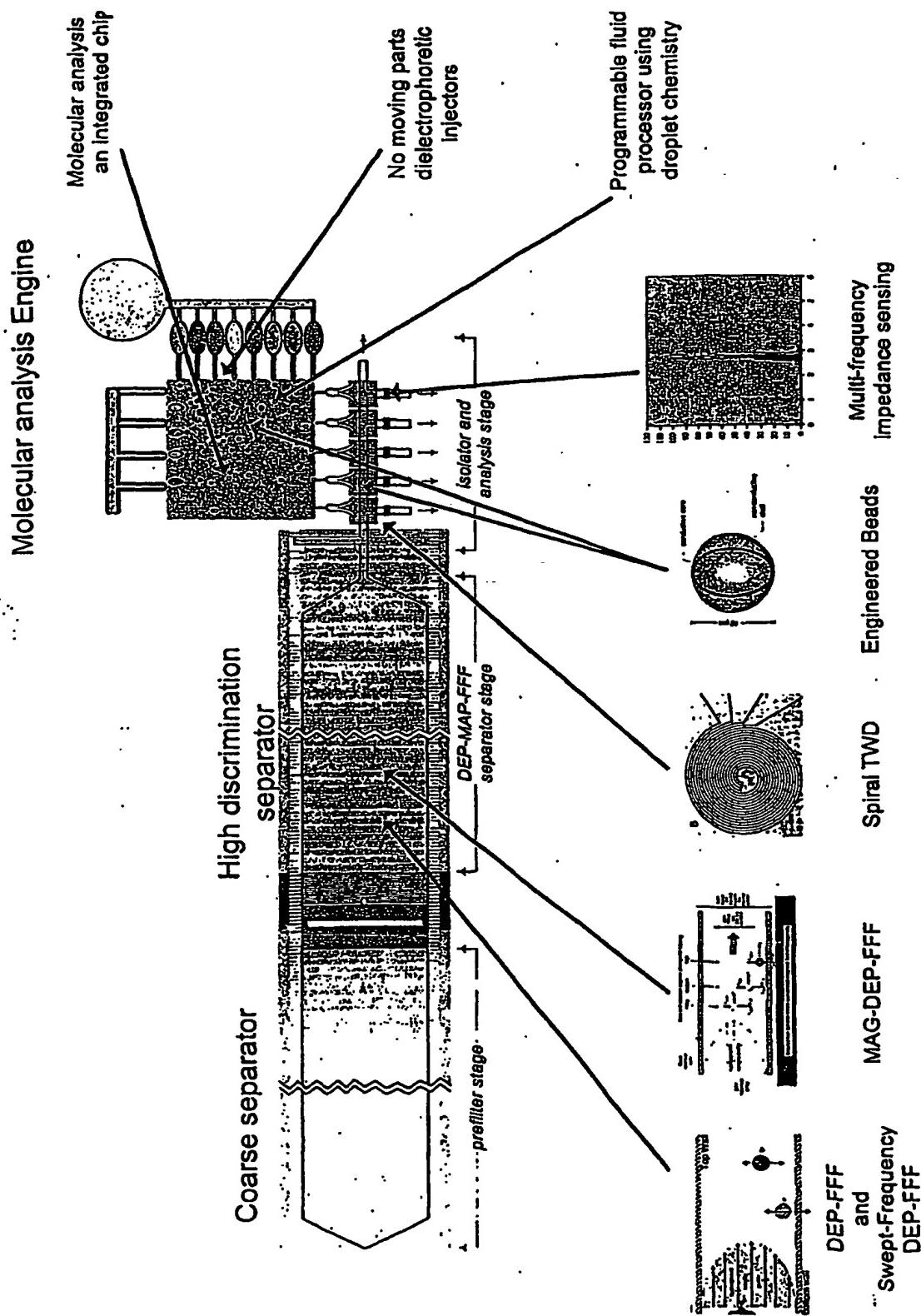


FIG. 11

FIG. 12





(19)

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(54) Mobile phase gradient generation microfluidic device

(57) The present invention relates to a microfluidic device (10) for separating the components of a fluid sample. A cover plate (40) is arranged over the first surface (14) of a substrate (12), which, in combination with a microchannel (18) formed in the first (14) surface, defines a separation conduit (19) for separating the components of the fluid sample. An inlet port (72) in fluid communication with the separation conduit (19) allows a mobile phase containing a gradient of a selected mobile-phase component to be introduced from an integrated gradient-generation means (60) to the separation conduit (19). A method is also provided for separating the components of a fluid sample using a mobile phase containing a gradient of a selected mobile-phase component, wherein the gradient is generated within a small volume of mobile phase.

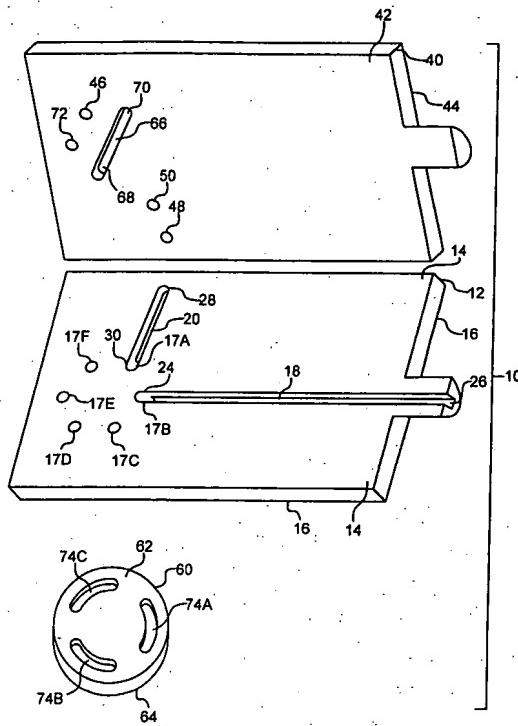


FIG. 1A

Description

[0001] The present invention generally relates to microfluidic devices that employ a mobile phase containing differing concentrations of a selected mobile-phase component. In particular, the invention relates to microfluidic devices that separate a fluid sample into its constituent components, wherein the employed mobile phase exhibits a concentration gradient of a selected mobile-phase component. In addition, the invention relates to the use of microfluidic devices to generate a gradient or differing concentrations of a selected mobile-phase component within a small volume of mobile phase in order to separate the components of a fluid sample.

[0002] Microfluidic device technologies, also referred to as lab-on-a-chip technologies, have been proposed for a number of different applications in various fields. In the field of biology, for example, microfluidic devices may be used to carry out cellular assays. In addition, microfluidic devices have been proposed to carry out separation techniques in the field of analytical chemistry.

[0003] Generally, microfluidic devices may be used to separate the components of a fluid sample using either of two techniques: capillary electrophoresis or chromatography. Capillary electrophoresis involves the separation of molecules based on differences in the electrophoretic mobility of the molecules. Typically, microfluidic devices employ a controlled application of an electric field to induce fluid flow and/or to provide flow switching. In order to effect reproducible and/or high-resolution separation, a fluid sample "plug" (a predetermined volume of fluid sample) must be controllably injected into a capillary separation column or conduit. For fluid samples containing high molecular weight charged biomolecular analytes, such as DNA fragments and proteins, microfluidic devices containing a capillary electrophoresis separation conduit that is a few centimeters in length may be effectively used to carry out separation of small sample plugs having a length on the order of micrometers. Once injected, high sensitivity detection, such as laser-induced fluorescence, may be employed to resolve a separated fluorescent-labeled sample component.

[0004] For samples containing analyte molecules with low electrophoretic mobility differences, such as those containing small drug molecules, the separation technology of choice is generally chromatography. Chromatographic separation occurs when a mobile phase carries sample molecules through a chromatography bed (stationary phase) where sample molecules interact with the stationary phase surface. The velocity at which a particular sample component travels through a chromatography bed depends on the component's partition between mobile phase and stationary phase. Microfluidic devices that incorporate a liquid chromatographic functionality have been described in U.S. Serial No.

09/908,231. These microfluidic devices may employ integrated mechanical valve technologies, such as those described in U.S. Serial No. 09/908,292, for sample introduction and to reduce the volume of "dead space" in the microfluidic devices.

[0005] There are many chromatographic techniques known in the art. See e.g., Kutter et al. (1998), "Solvent-programmed microchip open-channel electrochromatography," *Anal. Chem.* 70:3291-3297. For example, in reverse phase liquid chromatography, where the stationary phase offers a hydrophobic surface and the mobile phase is usually a mixture of water and an organic solvent, the least hydrophobic component moves through the chromatography bed first, followed by other components, in order of increasing hydrophobicity. In other words, the chromatographic separation of sample components may be based on hydrophobicity.

[0006] In isocratic liquid chromatography, the content of the mobile phase is constant throughout the separation. Gradient liquid chromatography, on the other hand, requires the content of the mobile phase to change during separation. Gradient liquid chromatography not only offers high resolution and high-speed separation of wide ranges of compounds, it also allows injection of large sample volumes without compromising separation efficiency. During the initial period when the sample is introduced, the mobile phase strength is often kept low, and the sample is trapped at the head of the liquid chromatography column bed. As a result, interfering moieties, such as salts, are washed away. In this regard, gradient liquid chromatography is suited to analyze fluid samples containing a low concentration of analyte moieties.

[0007] Typically, pressurizing means are employed to provide flow through packed columns in liquid chromatography. Such pressurizing means typically include pumps that are designed for optimal performance at a certain flow rate range, generally between about 50 µL/min to about 1 mL/min. To generate a gradient of a selected mobile-phase component within a mobile phase, two pumps may be employed to pump two fluids, each fluid containing a different concentration of the selected mobile-phase component. The fluids are mixed to form the mobile phase and introduced into the column. By varying the relative flow rate of the pumps, a concentration gradient of the selected mobile-phase component may be formed within the mobile phase flowing through the column. However, the quality of the gradient generated by this technique is limited by the performance of the pumps. In some cases, a gradient may require a fluid flow rate that lies outside the capability of the pumps. This limitation is particularly pronounced when microbore liquid chromatography columns are employed, because the required mobile-phase flow rate through the columns is extremely low.

[0008] Use of conventional chromatographic equipment with microfluidic devices for separating the components of a fluid may present other technical problems.

For example, in order to obtain a smooth gradient, conventional liquid chromatography systems employ a pressure damper as well as a mixer. The pressure damper and the mixer require a certain volume of fluid for proper operation. This volume is associated with a delay time, i.e., the time it takes for a mobile phase to reach the liquid chromatography column after mixing. The delay time can be calculated as the quotient of the delay volume over the flow rate. For example, the combined volume of the damper and the mixer in a conventional liquid chromatography system is about 0.3 mL to about 0.5 mL. This translates to a delay time of about 0.3 minutes to about 0.5 minutes at a flow rate of 1 mL/min. However, if a microfluidic device is constructed for operation at a flow rate of less than about 1 μ L/min, the delay time increases to about 300 minutes to about 500 minutes. Such a long delay time renders microfluidic device-based gradient liquid chromatography impractical.

[0009] To overcome this limitation, commercial low flow rate liquid chromatography pumps typically employ a split flow design. In this design, pumps move mobile phase at a high flow rate, but only a portion of the mobile phase is delivered to the separation column. The remainder of the mobile phase is diverted into a waste stream. This adds to the cost of operation because typically less than 1% of the mobile phase is actually used for separation.

[0010] In addition, the concentration of the selected mobile-phase components delivered to the column changes as gradient liquid chromatography is carried out. In most cases, the viscosity of the mobile phase also changes. This tends to change the pressure profile within the column. To compensate for such pressure profile changes, an electronic flow control unit that includes a flow meter and a variable flow resistor is used to control commercial chromatography pumps. As mobile phase is delivered to the column, the flow meter measures the flow rate of the mobile phase to provide feedback control over the variable flow resistor. Nevertheless, current flow sensor technologies are incapable of accurately measuring flow rates of less than 1 μ L/min, especially in gradient mode, and are therefore unsuitable for use in microfluidic devices that are employed in gradient chromatography.

[0011] Thus, there is a need for integrated microfluidic device technology that allows control and generation of a gradient of a selected mobile-phase component within a small volume of mobile phase in order to separate the components of a fluid sample. In particular, there is a need for an improved microfluidic device that employs a smooth gradient at low flow rates, especially in flow rate ranges of less than 1 μ L/min.

[0012] In a first embodiment, the invention relates to a microfluidic device for separating the components of a fluid sample. The microfluidic device comprises a substrate having a microchannel formed in a surface thereof and a cover plate arranged over the substrate surface. The cover plate, in combination with the microchannel,

defines a separation conduit for separating the components of the fluid sample according to a specific component property. The microfluidic device also includes an integrated gradient-generation means for generating a gradient of a selected mobile-phase component in a mobile phase. The integrated gradient-generation means is arranged to allow the mobile phase from the gradient-generation means to be transported through an inlet port into the separation conduit and out of an outlet port. Typically, though not necessarily, the integrated gradient-generation means is formed at least in part within the substrate and/or in the cover plate.

[0013] In another embodiment, the invention relates to a microfluidic device that employs a gradient-generation means for generating a gradient of a selected mobile-phase component in a mobile phase in order to separate the components of the fluid sample. The gradient-generation means is formed from a substrate and a cover plate arranged over a surface of the substrate. A microchannel having an upstream terminus and a downstream terminus is formed in the surface of the substrate. The cover plate, in combination with the microchannel, forms a mobile-phase holding conduit having a length defined by the upstream terminus and the downstream terminus. A plurality of inlet ports is arranged along the length of the mobile-phase holding conduit, and an outlet port is located downstream from the inlet ports. A separation conduit is provided as well for separating the components of a fluid sample according to a specific component property, and a mobile-phase introducing means allows mobile phase from the mobile-phase holding conduit to flow through its outlet port and into a separation conduit.

[0014] In a further embodiment, the invention relates to a microfluidic device having an integrated mobile-phase source. The microfluidic device comprises a substrate having a microchannel formed in a surface thereof and a cover plate arranged over the substrate surface. The cover plate, in combination with the microchannel, defines a separation conduit for separating the components of the fluid sample according to a specific component property, and has an inlet port and an outlet port. An integrated mobile-phase source is provided comprising a microconduit having a length defined by an upstream terminus and a downstream terminus. The microconduit contains a mobile phase that exhibits a gradient of a selected mobile-phase component along the length of the microconduit. The integrated mobile-phase source is arranged to allow the mobile phase to be transported through the inlet port into the separation conduit and out of the outlet port.

[0015] In a still further embodiment, the invention relates to a method for separating the components of a fluid sample. The method uses no more than about 100 μ L of fluid to produce a mobile phase containing a gradient of the selected mobile-phase component. Preferably, no more than about 20 μ L of fluid is used to produce the gradient-containing mobile phase. The gradient-

containing mobile phase produced as a result is used to convey a fluid sample through the separation conduit, thereby separating the components of the fluid sample.

[0016] In another embodiment, the invention relates to a microfluidic device for producing a flow of mobile phase. The device comprises a mobile-phase source and a pressurizing means. The mobile-phase source comprises a mobile-phase holding microconduit having a length defined by an upstream terminus and a downstream terminus, and an outlet port located at the downstream terminus, and a mobile phase, contained in the mobile-phase holding microconduit, that exhibits differing concentrations of selected mobile-phase component along the length of the mobile-phase holding microconduit. The pressurizing means allows for the pressurization of the microconduit to force the mobile phase within the microconduit to flow toward the downstream terminus along the length of the microconduit and out the outlet port.

[0017] In still another embodiment, the invention relates to a microfluidic device for producing a flow of mobile phase. The microfluidic device comprises, a producing means for producing different concentrations of a selected mobile-phase component in different locations within a mobile phase comprising, a plurality of mobile-phase sources, a mobile-phase introducing means and a pressurizing means. The producing means comprises a mobile-phase-holding microconduit having a length defined by an upstream terminus and a downstream terminus, an outlet port located at the downstream terminus, and at least one inlet port in fluid communication with the mobile-phase holding microconduit upstream from the outlet port. Each mobile-phase source contains a mobile phase of a different concentration of a selected mobile-phase component. The introduction means introduces plugs of mobile phase from the mobile-phase sources through the at least one inlet port into the mobile-phase holding conduit such that the plugs are arranged in a predetermined order along the length of the mobile-phase holding conduit. The pressurizing means pressurizes the microconduit to force the mobile phase within the microconduit to flow toward the downstream terminus along the length of the microconduit and out the single outlet port.

[0018] Thus, the invention provides, in yet another embodiment, a method for producing a flow of mobile phase. A mobile-phase source is provided comprising a mobile-phase-holding microconduit having a length defined by an upstream terminus and a downstream terminus, and an outlet port located at the downstream terminus, and a mobile phase, contained in the mobile-phase holding microconduit, that exhibits differing concentrations of selected mobile-phase component along the length of the microconduit. The mobile-phase holding microconduit is pressurized to force the mobile phase within the mobile-phase holding microconduit to flow toward the downstream terminus along the length of the microconduit and out of the outlet port. Optionally,

at least one inlet port is provided fluid communication with the mobile-phase holding microconduit, wherein the outlet port is located downstream from the at least one inlet port of the mobile-phase holding microconduit.

5 In such a case, a plurality of mobile-phase sources may be provided as well, each containing a mobile phase, wherein each mobile phase contains a different concentration of a selected mobile-phase component. Plugs of mobile phase from the mobile-phase sources are introduced through the at least one inlet port into the mobile-phase holding microconduit such that the plugs are arranged in a predetermined order along the length of the mobile-phase holding conduit.

[0019] For any of the embodiments, the mobile-phase holding microconduit may be defined or further defined by a substrate having a microchannel formed in a surface thereof in combination with a cover plate arranged over the substrate surface.

[0020] A number of preferred embodiments of the invention will now be described with reference to the drawings, in which:-

FIGS. 1A-1C, collectively referred to as FIG. 1, illustrate a version of the inventive microfluidic device having an integrated gradient-generation means. FIG. 1A illustrates the device in exploded view. FIGS. 1B and 1C schematically illustrate the microfluidic device in loading and operating configurations, respectively.

25 FIGS. 2A-2C, collectively referred to as FIG. 2, illustrate a version of the inventive microfluidic device similar to that illustrated in FIG. 1, but having a different integrated gradient-generation means. FIGS. 2A and 2B schematically illustrate the microfluidic device in loading configurations. FIG. 2C schematically illustrates the microfluidic device in an operating configuration.

30 FIG. 3 schematically illustrates a microfluidic device for separating the components of a fluid sample, wherein the microfluidic device contains a cascade of microchannels suitable for generating a smooth gradient in a mobile phase.

35 FIGS. 4A-4C, collectively referred to as FIG. 4, illustrate a switching structure employs rotational motion to controllably introduce, through a conduit, a plug of a first mobile phase followed by a second mobile phase. FIG. 4A illustrates the switching structure in exploded view. FIGS. 4B and 4C schematically illustrate the switching structure in first and second flow path configurations, respectively.

40 FIGS. 5A and 5B, collectively referred to as FIG. 5, illustrate a switching structure that employs rotational motion to controllably introduce, through a conduit, a plug of a first mobile phase, followed by a plug of a second mobile phase and the first mobile phase. FIG. 5A illustrates the switching structure in a first flow path configuration. FIG. 5B illustrate the switching structure in a second flow path configura-

tion.

[0021] Before the invention is described in detail, it is to be understood that, unless otherwise indicated, this invention is not limited to particular materials, components, or manufacturing processes, as such may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting.

[0022] It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an inlet" includes a plurality of inlets, reference to "a fluid" includes a mixture of fluids, reference to "a cascade" includes a plurality of cascades, and the like.

[0023] In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings:

[0024] The term "fluid-tight" is used herein to describe the spatial relationship between two solid surfaces in physical contact such that fluid is prevented from flowing into the interface between the surfaces.

[0025] The term "fluid-transporting feature" as used herein refers to an arrangement of solid bodies or portions thereof that direct fluid flow. Fluid-transporting features include, but are not limited to, chambers, reservoirs, conduits, and channels. The term "conduit" as used herein refers to a three-dimensional enclosure formed by one or more walls and having an inlet opening and an outlet opening through which fluid may be transported. The term "channel" is used herein to refer to an open groove or a trench in a surface. A channel in combination with a solid piece over the channel forms a "conduit". A conduit may also be formed from a stencil interposed between two solid surfaces.

[0026] The term "microalignment means" is defined herein to refer to any means for ensuring the precise microalignment of microfabricated features in a microfluidic device. Microalignment means can be formed either by laser ablation or by other methods well known in the art that are used to fabricate shaped pieces. Representative microalignment means that can be employed herein include a plurality of appropriately arranged protrusions in component parts, e.g., projections, depressions, grooves, ridges, guides, or the like.

[0027] The term "microfluidic device" refers to a device having features of micrometer or submicrometer dimensions, and which can be used in any number of chemical processes involving very small amounts of fluid. Such processes include, but are not limited to, electrophoresis (e.g., capillary electrophoresis or CE), chromatography (e.g., μ LC), screening and diagnostics (e.g., using hybridization or other binding means), and chemical and biochemical synthesis (e.g., DNA amplification as may be conducted using the polymerase chain reaction, or "PCR"), and analysis (e.g., through enzymatic digestion). The features of the microfluidic devices

are adapted to the particular use intended. For example, microfluidic devices that are used in separation processes such as chromatography contain microchannels (termed herein as "microconduits" when they are enclosed, i.e., when the cover plate is in place on the microchannel-containing substrate surface) on the order of 1 μ m to 200 μ m in diameter, typically 10 μ m to 75 μ m in diameter, and approximately 0.1 to 50 cm in length. For microfluidic devices that are used in generating or

holding a gradient-containing mobile phase, conduits associated with gradient-generation means may have a volume of about 1 nL to about 100 μ L, typically about 10 nL to 20 μ L.

[0028] The term "mobile phase" as used herein refers to any fluid capable of movement under a motive force. Although the term "mobile phase" is typically used in the context of separation processes such as chromatography, the term is not limited to such processes.

[0029] "Optional" or "optionally" as used herein means that the subsequently described feature or structure may or may not be present, or that the subsequently described event or circumstance may or may not occur, and that the description includes instances where a particular feature or structure is present and instances where the feature or structure is absent, or instances where the event or circumstance occurs and instances where it does not.

[0030] The invention generally relates to a microfluidic device for separating the components of a fluid sample, wherein the microfluidic device is formed from a substrate having a microchannel in a surface thereof and a cover plate arranged over the substrate surface. The cover plate, in combination with the microchannel, defines a separation conduit for separating the components of a fluid sample according to a specific component property; the separation conduit has an inlet port and an outlet port. The microfluidic device also includes an integrated gradient-generation means for generating a gradient of a selected mobile-phase component in a mobile phase. The integrated gradient-generation means is arranged to allow the mobile phase from the gradient-generation means to be transported through the inlet port into the separation conduit and out of the outlet port. Typically, though not necessarily, the integrated gradient-generation means is formed at least in part within the substrate and/or in the cover plate.

[0031] Typically, the integrated gradient-generation means includes a mobile-phase holding conduit having a length defined by an upstream terminus and a downstream terminus. A plurality of inlet ports is arranged along the length of the mobile-phase holding conduit, and an outlet port is located downstream from the inlet ports of the mobile-phase holding conduit. An introduction means is provided for introducing the mobile phase from the mobile-phase holding conduit through its outlet port and into the inlet port of the separation conduit. This gradient-generation means, as well as variations thereof, is further discussed below.

[0032] FIG. 1 illustrates an embodiment of the inventive microfluidic device having an integrated gradient-generation means as described above, in combination with a separation column for liquid chromatography. As with all figures referenced herein, in which like parts are referenced by like numerals, FIG. 1 is not necessarily to scale, and certain dimensions may be exaggerated for clarity of presentation. As illustrated in FIG. 1, the microfluidic device 10 includes a substrate 12 comprising first and second substantially planar opposing surfaces indicated at 14 and 16, respectively, and is comprised of a material that is substantially inert with respect to fluids that will be transported through the microfluidic device. The substrate 12 has a first fluid-transporting feature in the form of a separation microchannel 18 and a second fluid-transporting feature in the form of a mobile-phase holding microchannel 20, each microchannel located in the first planar surface 14. The separation microchannel 18 represents a portion of a separation conduit 19, and the mobile-phase holding microchannel 20 represents a portion of the mobile-phase holding conduit 21, as discussed below. The separation microchannel 18 has a sample inlet terminus 24 at an upstream end and a sample outlet terminus 26 at the opposing end. As shown in FIG. 1, the sample outlet terminus 26 is located at a protrusion of the otherwise rectangular substrate 12. Similarly, the mobile-phase holding microchannel 20 has a length defined by an upstream terminus 28 and a downstream terminus 30.

[0033] The substrate 12 has six conduits, indicated at 17A, 17B, 17C, 17D, 17E and 17F that extend through surfaces 14 and 16 and that represent the vertices of an equilateral hexagon. Conduit 17A is located at the downstream terminus 30 of the mobile-phase holding microchannel 20. Conduit 17B is located at the sample inlet terminus 28 of the separation microchannel 18.

[0034] The microfluidic device 10 also includes a cover plate 40 that is complementarily shaped with respect to the substrate 12 and has first and second substantially planar opposing surfaces indicated at 42 and 44, respectively. The cover plate 40 can be comprised of any suitable material for forming the substrate 12 as described below. The contact surface 42 of the cover plate 40 is capable of interfacing closely with the contact surface 14 of the substrate 12 to achieve fluid-tight contact between the surfaces. The cover plate 40 may include a variety of features. As shown, mobile-phase inlet ports 48 and 50 are provided as a cylindrical conduit extending through the cover plate 40 in a direction orthogonal to the cover plate contact surface 42 to result in fluid communication between surfaces 42 and 44. Similarly, sample inlet port 72 and sample outlet port 46 are provided as conduits extending from surface 42 to surface 44. A sample introduction channel 66 having termini 68 and 70 is located on contact surface 42.

[0035] The cover plate 40 is substantially immobilized over the substrate contact surface 14, and the cover plate contact surface 42 in combination with the sepa-

ration microchannel 18 defines a sample conduit 19 for conveying the sample. Similarly, the cover plate 40, in combination with the mobile-phase holding channel 20, defines a mobile-phase holding conduit 21. In addition, the sample introduction channel 66 combined with the substrate contact surface 14 forms sample introduction conduit 67. Because the contact surfaces of the cover plate and the substrate are in fluid-tight contact, the separation conduit 19, the mobile-phase holding conduit 21, and sample introduction conduit 67 are all fluid-tight as well. Mobile-phase inlet port 48 is located over the upstream terminus 28 of the mobile-phase holding-channel 20 and mobile phase inlet port 50 is located over the mobile-phase holding channel 20 at approximately the midpoint between its termini 28 and 30. Sample inlet port 72 and sample outlet port 46 are aligned with conduits 17E and 17D, respectively. Termini 68 and 70 of sample introduction channel 66 are aligned with conduits 17F and 17C, respectively.

[0036] A switching plate 60 is provided as an integrated means for sample introduction. The switching plate also serves to provide controllable communication between the mobile-phase holding conduit 21, the sample introduction conduit 67, and the separation conduit 19.

This switching plate 60 is similar to that described in U. S. Serial Nos. 09/908,292 and 09/908,231. As depicted in FIG. 1A, the switching plate 60 has a substantially planar and circular contact surface 62 and an opposing surface 64. As shown, the surfaces 62 and 64 are generally congruent. Three curved fluid-transporting channels, indicated at 74A, 74B and 74C, are each located on contact surface 62. The three fluid-transporting channels lie along a circle having a diameter equal to the length of sample introduction channel 66. Each fluid-transporting channel has two termini such that each terminus represents a vertex of an equilateral hexagon that is congruent with the equilateral hexagon formed by the through conduits 17A-17F of the substrate 12. The switching plate contact surface 62 may be placed in slidable and fluid-tight contact with substrate surface 16. As a result, the fluid-transporting channels, 74A, 74B, and 74C, in combination with substrate surface 16, form three curved conduits, 75A, 75B, and 75C, respectively.

[0037] Depending on the relative orientation of the switching plate and the substrate two possible flow paths configurations can be created. As shown in FIG. 1B, the first flow path configuration allows a sample fluid to be introduced into the microfluidic device through sample inlet port 72 through conduit 75C, the sample introduction conduit 67 and conduit 75B. The sample fluid may then exit the microfluidic device 10 through sample outlet port 46. By rotating the switching plate 60 60° about its center, a second flow path configuration results, as shown in FIG. 1C. The second flow path configuration positions the sample introduction conduit 67 in a flow path between the mobile-phase holding conduit 21 and the separation conduit 19. As a result, a flow path is formed from mobile-phase holding conduit 21 through

conduit 75C, sample introduction conduit 67, conduit 75A, and separation conduit 19.

[0038] The microfluidic device may be employed in a method for separating the components of a fluid sample in a manner similar to that of a simple capillary liquid chromatographic apparatus. The integrated gradient-generation means may be used to generate the gradient of the selected mobile-phase component in the mobile phase by providing the inlet ports 48 and 50 of the mobile-phase holding conduit fluid communication with a source of mobile phase (not shown) such that mobile phase containing differing concentrations of the selected mobile-phase component may be introduced into each inlet port. Thus, once filled with mobile phases from the inlet ports 48 and 50, the upstream portion of the mobile-phase holding conduit 21 may contain a different concentration of the selected mobile-phase component than the downstream portion. Given sufficient time for diffusion to occur, the mobile-phase holding conduit 21 along its length may contain mobile phase that exhibits a smooth gradient of the selected mobile-phase component. By proper choice of the concentration of the selected mobile-phase component in a mobile-phase source, the gradient of the selected mobile-phase component may increase or decrease along the length of the mobile-phase holding conduit 21 in a downstream direction.

[0039] In addition, as shown in FIG. 1B, fluid sample is introduced into the sample inlet port 72 to fill the sample introduction conduit 67. Typically, the combination of the sample introduction conduit 67 and conduit 75C holds a predetermined volume (i.e., a plug) of sample therein. Once a desired gradient is formed in the mobile-phase holding conduit 21, the switching plate 60, as indicated in FIG. 1C is slidably rotated to provide communication between the mobile-phase holding conduit 21 and the separation conduit 19. A pressurizing means (not shown) provides a motive force to deliver the mobile phase contained in the mobile-phase holding conduit 21 through the sample introduction conduit 67 and into the separation conduit 19. As a result, the fluid sample contained within the sample introduction conduit 67 is conveyed though the separation conduit 19. Typically, separation within the separation conduit 19 is carried out using a mobile-phase flow rate of no more than about 1 $\mu\text{L}/\text{min}$. However, flow rates of about 0.01 $\mu\text{L}/\text{min}$ to about 10 $\mu\text{L}/\text{min}$ may be employed, with flow rates of about 0.1 $\mu\text{L}/\text{min}$ to about 2 $\mu\text{L}/\text{min}$ preferred. The fluid sample is then separated into sample components according to a specific component property and emerges from an outlet port 47 at the terminus 26 of the separation conduit. The outlet port 47 may be interfaced with a collector, such as a sample vial, plate, or capillary. The collector may serve as a storage device or represent an intermediary to another device that uses and/or analyzes collected sample fractions. Alternatively, an analytical device such as a mass spectrometer may be directly or indirectly interfaced with the outlet port 47 for fraction

analysis.

[0040] Although a plurality of inlet ports is depicted as arranged along the length of the mobile-phase holding conduit in FIG. 1, this configuration is not a necessity. FIG. 2 illustrates another version of the inventive microfluidic device 10, similar to that depicted in FIG. 1 except that the mobile-phase holding conduit 21 of the integrated gradient-generation means has a single inlet port 48 located at its upstream terminus rather than a plurality of inlet ports arranged along the length of the mobile-phase holding conduit. In addition, the integrated gradient-generation means includes an additional switching plate 80 that contains a mobile-phase introduction conduit 82 to provide the mobile-phase holding conduit 21 with alternating fluid communication with a plurality of mobile-phase sources indicated at 49 and 51, and each source containing a different concentration of the selected mobile-phase component for gradient formation. As shown in FIG. 2A, a first mobile-phase source 49 is provided fluid communication through the mobile-phase introduction conduit 82 with the mobile-phase holding conduit 21 of the microfluidic device 10, while a second mobile-phase source 51 is fluidly isolated from the mobile-phase holding conduit 21. FIG. 2B illustrates that the second mobile-phase source 51 may be provided fluid communication with the mobile-phase holding conduit 21 through the mobile-phase introduction conduit 82 while the first mobile-phase source 49 is fluidly isolated from the mobile-phase holding conduit 21.

[0041] By providing the mobile-phase holding conduit 21 with alternating fluid communication in a predetermined loading sequence with mobile-phase sources 49 and 51, the mobile-phase holding conduit 21 may be filled with a mobile phase having a concentration profile of the selected mobile-phase component along the length of the mobile-phase holding conduit 21. As before, given sufficient time for diffusion to occur, the mobile phase along the length of the mobile-phase holding conduit 21 may come to contain a smooth gradient of the selected mobile-phase component. For example, the first mobile-phase source 49 may contain the selected mobile-phase component in a pure form, and the second mobile-phase source 51 may contain a fluid that does not contain the selected mobile-phase component. Plugs of increasing volume of fluid from the first mobile-phase source 49 are alternatingly introduced through inlet port 48 into the mobile-phase holding conduit 21 with plugs of identical volumes of fluid from the second mobile-phase source 51. Such alternating introduction can be effected through a number of valve actuation technologies including those that employ computer-aided control. After sufficient time has elapsed to allow for diffusion of the selected mobile-phase component, the mobile-phase holding conduit 21 will exhibit a smooth and increasing gradient of the selected mobile-phase component along its length in an upstream direction, as indicated by arrow A. Once the desired gradient is formed in the mobile-phase holding conduit 21, the mobile

phase may be delivered into a separation conduit 19 in order to separate the components of a sample, as illustrated in FIG. 2C.

[0042] In another embodiment, the invention relates to a microfluidic device for separating the components of a fluid sample. The microfluidic device comprises a gradient-generation means, a separation conduit for separating the components of a fluid sample according to a specific component property, as well as a means for introducing mobile phase from the gradient-generation means. The gradient-generation means is constructed from a substrate and a cover plate. A microchannel is formed in a surface of the substrate and has an upstream terminus and a downstream terminus. The cover plate is arranged over the substrate surface and, in combination with the microchannel, forms a mobile-phase holding conduit having a length defined by the upstream terminus and the downstream terminus. A plurality of inlet ports is arranged along the length of the mobile-phase holding conduit, and an outlet port is located downstream from the inlet ports of the mobile-phase holding conduit. Each of the inlet ports may fluidly communicate with the same or different sources of mobile phase as described below.

[0043] Generally, increasing the number of inlet ports for the mobile-phase holding conduit will provide greater control in gradient generation. Thus, the mobile-phase holding conduit may have any number of inlet ports greater than two. It is generally preferred that the inlet ports be evenly spaced along the length of the mobile-phase holding conduit, particularly when the mobile-phase holding conduit represents a gradient-generation means that comprises a cascade of conduits that split and mix streams of liquids (for example, water and methanol). A similar cascade is generally described in Whitesides et al. (2001), "Flexible Methods for Microfluidics," *Physics Today* 52(6):42-47. As described in Whitesides et al., the cascade may be employed in techniques that involve the immobilization of cells in a pattern on a surface of a microfluidic device wherein the cells are exposed to fluids containing appropriate reagents. Splitting of streams of liquid occurs at T-junctions between mixing channels and distribution channels. At steady state, the ultimate result of this splitting and mixing is the generation of a stepwise concentration gradient. However, the fluids may be delivered as a linear gradient of an appropriate reagent, e.g., a dye gradient, formed by diffuse mixing through a network of microchannels.

[0044] FIG. 3 illustrates an example of such a cascade on a microfluidic device 10 similar to that illustrated in FIG. 1. The cascade is depicted as a collection of microconduits formed from the combination of microchannels on a substrate and a cover plate, although this is not a necessity. The gradient-generation means includes a mobile-phase holding conduit 21 having an upstream terminus 28 and a downstream terminus 30. Five inlet ports, indicated at 48A, 48B, 48C, 48D, and 48E,

are evenly spaced along its length. An outlet port 52 is located at the downstream terminus 30. As shown, no inlet port is located at either terminus of the mobile-phase holding conduit. Three distribution conduits, indicated at 90, 100, and 110, are arranged in parallel disposition in successively increasing distances to the mobile-phase holding conduit 21. Each successive distribution conduit from the mobile-phase holding conduit 21 has the same number of outlet ports as the preceding conduit. However, each successive distribution conduit from the mobile-phase holding conduit 21 has one fewer inlet port than the preceding conduit. Thus, distribution conduit 90 has five outlet ports 91A, 91B, 91C, 91D, and 91E, and four inlet ports 92A, 92B, 92C, and 92D; distribution conduit 100 has four outlet ports 101A, 101B, 101C, and 101D, and three inlet ports 102A, 102B, and 102C; and distribution conduit 110 has three outlet ports 111A, 111B, and 111C, and two inlet ports 112A and 112B. Each outlet port of each distribution conduit fluidly communicates via a mixing conduit with an inlet port of a successive conduit. Each inlet port of the distribution conduits is located between two outlet ports of the same distribution conduit.

[0045] The gradient-generation process is now described. Turning to distribution conduit 110, inlet ports 112A and 112B are provided fluid communication with two sources of mobile phase, each holding a different concentration of the selected mobile-phase component for which a gradient is to be generated. Typically, one of the mobile-phase sources (in this case, the mobile-phase source communicating with inlet port 112A, holds a first mobile phase of the selected mobile-phase component while the other mobile-phase source (in this case, the mobile-phase source communicating with inlet port 112B holds a second mobile phase that contains another selected mobile-phase component altogether. When mobile phases from the mobile-phase sources are introduced into distribution conduit 110, the first and second mobile phases will preferentially fill the portion of the conduit closest to their associated inlet ports. Thus, the first mobile phase will preferentially fill the portion of distribution conduit 110 closer to outlet port 111A, and the second mobile phase will preferentially fill the portion of the distribution conduit 110 closer to terminus 111C. Mixing channel 115A will contain only the first mobile phase, and mixing channel 115C will contain only the second mobile phase.

[0046] However, as mixing channel 115B lies between inlet ports 112A and 112B, mixing channel 115B will contain both mobile phases. Thus, the inlets along distribution conduit 100 will provide mobile phases containing increasing concentrations the selected mobile phase along the length of the distribution conduit from outlet 101A to outlet 101D. It should be evident that this mixing and redistribution process is repeated for each of the distribution conduits to result in the generation of a smooth gradient in the mobile-phase holding conduit 21. Optionally, a plurality of waste/exhaust ports indicat-

ed at 121 is provided in fluid communication with mobile-phase holding conduit to allow any air or other fluid contained in the mobile-phase holding conduit to be displaced during the gradient generation process. As shown, the waste/exhaust ports may fluidly communicate with each other through converging conduits 123 at gradient outlet 125. Once a gradient is generated in the mobile-phase holding conduit 21, a pressurizing means (not shown) may be employed to provide a motive force to deliver the mobile phase contained in the mobile-phase holding conduit into the separation conduit 19 to carry out separation processes as described above and elsewhere herein.

[0047] In order to ensure the proper formation of a smooth gradient, the following considerations should be addressed. First, the residence time of the fluids in the mixing conduits should be sufficiently long to ensure near-complete mixing. Second, the flow resistance of the mixing conduits should be significantly greater than the flow resistance of the distribution and holding conduits. This minimizes backflow into the mixing conduits as the concentration gradient is pumped out of the holding conduit. Third, the characteristic length of the cascade should be long enough to prevent destruction of the gradient by complete diffuse mixing while it is waiting to be pumped into the separations. However, as discussed above, some degree of diffusion may be beneficial in smoothing of the concentration steps. Finally, additional design considerations, such as tailoring of the dimensions of each individual mixing conduit, can be made to account for differences in viscosity between the two mobile phases. Although mobile phases having different viscosities may be used, the following analyses assume that the mobile phases have the same viscosity and that the conduits are formed from conduits.

[0048] With respect to residence time, a two-dimensional order of magnitude analysis assuming a uniform flow with no entrance length effects and no reflections at the conduit, yields the following dependence for the characteristic mixing length, L_m :

$$L_m \sim a^2 \bar{u} / 2D = a \cdot Pe / 2 \quad (1)$$

where a is the half-width of the conduit, \bar{u} is the average flow velocity in the conduit, D is the diffusion coefficient of the diffusing species, and Pe is the Peclet number (which expresses the ratio of convective to diffusive transport).

[0049] The gradient-generation cascade is designed such that the flow resistance of the mixing conduits is much greater than the flow resistance of the distribution and holding conduits. In this case, the flow resistance of the distribution conduits can be neglected, resulting in an equal distribution of the total volumetric flow rate, Q , among the mixing conduits. Each mixing conduit would then support a flow rate equal to the total flow

rate, Q , divided by the number of conduits in that stage of the cascade. Thus, the highest flow rates are encountered at the first mixing conduits, resulting in the following order of magnitude estimate for the characteristic mixing length of conduits with a cross-sectional area given by $2a * h$ (twice the half-width, multiplied by the depth of the conduit):

$$L_m \sim aQ / 12Dh \quad (2)$$

[0050] Then, for example, the characteristic mixing length for a species with a diffusion coefficient of $1.28 \times 10^{-5} \text{ cm}^2/\text{s}$, a conduit with a depth $h = a$, and a total volumetric flow rate of 300 nL/min , would be on the order of $330 \mu\text{m}$. In summary, the mixing conduits should be at least three times the characteristic mixing length, which is directly proportional to the total volumetric flow rate and inversely proportional to the product of the diffusion coefficient and the aspect ratio, h/a , of the conduit. For a given diffusing species, we can reduce the mixing length by lowering the total flow rate and aspect ratio of the conduit.

[0051] A more detailed analytical solution for the two-dimensional problem, including wall reflections, is given in Jeon et al. (2000), "Generation of solution and surface gradients using microfluidic systems," *Langmuir* 16: 8311-8316; and in Crank, *The Mathematics of Diffusion*, 2nd Ed., Oxford University Press, Oxford, 1975, pp. 16. The Netflow Module of FlumeCAD (Coventor, Inc., Cary, NC) has also been employed to calculate the mixing length for the conditions listed above. According to these simulations, near-complete mixing occurs at approximately 1000 mm into the conduit, or approximately three characteristic mixing lengths away from the entrance to the conduit.

[0052] With respect to the flow resistance of the mixing conduits in relationship to the flow resistance of the distribution and holding conduits, the equivalent flow resistance of a flow conduit supporting fully developed laminar flow can be approximated by:

$$R = |\Delta p/Q| = 128 \mu L / \pi d_h^4 \quad (3)$$

where Δp is the change in pressure, μ is the viscosity of the fluid, L is the length of the conduit, and d_h is the hydraulic diameter of the conduit. The hydraulic diameter is defined as four times the cross-sectional area of the conduit, divided by the perimeter of the cross section. The depth, h , of a shallow conduit dominates in the computation of the hydraulic diameter.

[0053] The requirement that the flow resistance of the mixing conduits be much greater than the flow resistance of the distribution and holding conduits can be expressed as:

$$(d_h)_{\text{dist}}^4/L_{\text{dist}} >> (d_h)_{\text{mix}}^4/L_{\text{mix}} \quad (4)$$

[0054] The characteristic length of the cascade, A, and the diffusion coefficient of the diffusing species are the parameters that determine the stability of the gradient in the holding conduit as the gradient is waiting to be introduced into the separation conduit. From a simulation of the concentration of water and methanol in the holding conduit as a function of time for a conduit 200 μm wide, a characteristic length of 5000 μm , and nine gradient steps (i.e., a gradient cascade of order 9), a smooth gradient is formed after about one minute and is stable for at least 50 minutes.

[0055] Thus, the invention also relates to a microfluidic device having an integrated mobile-phase source. The microfluidic device comprises a substrate having a microchannel formed in a surface thereof and a cover plate arranged over the substrate surface. The cover plate in combination with the microchannel defines a separation conduit for separating the components of a fluid sample according to a specific component property and has an inlet port and an outlet port. An integrated mobile-phase source is provided, which comprises a microconduit having a length defined by an upstream terminus and a downstream terminus. The microconduit contains a mobile phase that exhibits a gradient of a selected mobile-phase component along the length of the microconduit. The integrated mobile-phase source is arranged to allow the mobile phase to be transported through the inlet port into the separation conduit and out of the outlet port.

[0056] Through proper microfluidic device design and construction, it is now possible to use no more than about 100 μL of fluid to produce a mobile phase containing a gradient of the selected mobile-phase component to carry out gradient chromatography. Preferably, no more than about 50 μL of fluid is used to produce the gradient-containing mobile phase. Optimally, no more than about 10 μL of fluid is used to produce the gradient-containing mobile phase. The gradient-containing mobile phase is used to convey a fluid sample through the separation conduit, thereby separating the components of the fluid sample.

[0057] From the above, it is evident that the invention, in addition to providing a device for separating the components of a fluid, also provides a microfluidic device for producing a flow of mobile phase. The device comprises a mobile-phase source and a pressurizing means. The mobile-phase source comprises a mobile-phase holding microconduit having a length defined by an upstream terminus and a downstream terminus, and an outlet port located at the downstream terminus, and a mobile phase, contained in the mobile-phase holding microconduit, that exhibits differing concentrations of selected mobile-phase component along the length of the mobile-phase holding microconduit. The pressuriz-

ing means allows for the pressurization of the microconduit to force the mobile phase within the microconduit to flow toward the downstream terminus along the length of the microconduit and out the outlet port. Any number of pressurizing means known in the art may be used and include, for example, syringes, pumps, pressurized gases, etc. Although electrokinetic forces may be used as well, they are not preferred. Such forces are dependent on the concentration of the mobile phase and the electrokinetic mobility will vary along any flow path exhibiting a gradient, leading to difficulties in control over mobile phase movement:

[0058] The mobile-phase as described above, may be produced by any of a number of different techniques, described herein with references to FIGS. 1-3, in the context of chemical separation using a gradient-containing mobile phase. However, mobile phases may also be employed in the context of fields other than chemical separation. In addition, it should be apparent the gradient-generation means as described above may be adapted to produce a mobile phase that contains different concentrations of a selected mobile-phase component at different locations within a mobile phase.

[0059] Thus, the invention also generally relates to a microfluidic device for producing a flow of mobile phase, wherein a producing means is provided to generate a gradient of a selected mobile-phase component in a mobile phase, to produce a mobile phase exhibiting different concentration of a selected mobile-phase component in different locations within the mobile phase, or both. Such a producing means typically comprises a mobile-phase-holding microconduit having a length defined by an upstream terminus and a downstream terminus, an outlet port located at the downstream terminus, and at least one inlet port in fluid communication with the mobile-phase holding microconduit upstream from the outlet port. A plurality of mobile-phase sources are also provided, wherein each source contains a mobile phase having a different concentration of a selected mobile-phase component. An introduction means introduces plugs of mobile phase from the mobile-phase sources through the at least one inlet port into the mobile-phase holding conduit such that the plugs are arranged in a predetermined order along the length of the mobile-phase holding conduit. As illustrated in FIG. 2, the introduction means may be formed at least in part by a switching structure.

[0060] FIGS. 4 and 5 illustrate two additional switching structures that may be employed as an introduction means. FIG 4 illustrates a microfluidic switching structure similar to that described in U.S. Serial No. 09/908,231. The switching structure employs rotational motion to controllably introduce a plug of a mobile phase. As illustrated in FIG. 4A, the switching structure includes a substrate 12 comprising first and second substantially planar opposing surfaces indicated at 14 and 16, respectively. The substrate 12 has six conduits, indicated at 17A, 17B, 17C, 17D, 17E and 17F that ex-

tend through surfaces 14 and 16 and that represent the vertices of an equilateral hexagon.

[0061] The switching structure 10 also includes a cover plate 40 that is complementarily shaped with respect to the substrate 12 and has first and second substantially planar opposing surfaces indicated at 42 and 44, respectively. The contact surface 42 of the cover plate 40 is capable of interfacing closely with the contact surface 14 of the substrate 12 to achieve fluid-tight contact between the surfaces. The cover plate 40 is substantially immobilized over the substrate contact surface 14. The cover plate 40 includes a variety of features. As shown, a first cover plate conduit 48A is provided extending through the cover plate in a direction orthogonal to the cover plate contact surface 42 to provide communication between surfaces 42 and 44. The first cover plate conduit 48A is arranged to communicate with the conduit 17A of the substrate 12 and enables passage of first mobile phase from a first mobile phase source (not shown) through conduit 17A to communicate with switching plate 120 as discussed below. Two additional cylindrical conduits, indicated at 53A and 48B are provided fluid communication with conduits 17F and 17C, respectively.

[0062] A linear channel 53B having two termini, indicated at 53C and 53D, is located in contact surface 42. The termini 53C and 53D fluidly communicate with conduits 17E and 17B, respectively. The termini 53C and 53D in combination with conduits 48A, 48B and 53A represent five of six vertices of an equilateral hexagon. Accordingly, each of the conduits is located the same distance from the center point of the channel 53B. As discussed above, the cover plate 40 is substantially immobilized over the substrate contact surface 14. As a result, substrate surface 14 in combination with channel 53B forms a conduit 55, which serves as a plug holding chamber, discussed below. Alternatively, the linear channel 53B may be provided on substrate surface 14. In such a case, termini 53C and 53D would coincide in location with conduits 17B and 17E, respectively.

[0063] As shown in FIG. 4A, the switching plate 120 has a substantially planar and circular contact surface 122 and an opposing surface 124. Three curved fluid-transporting channels, indicated at 126, 128 and 130, are each located on contact surface 122. The three fluid-transporting channels lie along a circle having a diameter equal to the length of channel 53B. Each fluid-transporting channel has two termini: termini 126A and 126B are associated with channel 126, termini 128A and 128B are associated with channel 128, and termini 130A and 130B are associated with channel 130. The switching plate contact surface 122 may be placed in slidably and fluid-tight contact with substrate surface 16. As a result, the fluid-transporting channels, 126, 128 and 130, in combination with substrate surface 16, form three curved conduits; 126C, 128C, 130C, respectively.

[0064] Depending on the relative orientation of the switching plate and the substrate, at least two possible

flow paths configurations can be created. As shown in FIG. 4B, the first flow path configuration allows a first mobile phase from a first mobile-phase source to travel, in order, through conduit 48A, conduit 17A, conduit 126C, conduit 17B, conduit 55, conduit 17E, conduit 128C, conduit 17F and conduit 53A. The first flow path configuration also allows a second mobile phase from a second mobile-phase source (not shown) to travel, in order, through conduit 48B, conduit 17C, conduit 130C, and conduit 17D. Typically, the first and second mobile-phase sources contain differing concentrations of a mobile phase component. By rotating the switching plate 120 60° about its center, a second flow path configuration results, as shown in FIG. 4C. The second flow path configuration allows the first mobile phase to travel, in order, through conduit 48A, conduit 17A, conduit 126C, conduit 17F, and conduit 453A. In addition, the second flow path configuration allows the second mobile phase to travel, in order, through conduit 48B, conduit 17C, conduit 130C, conduit 17B, conduit 55, conduit 17E, conduit 128C and conduit 17D.

[0065] In use, the switching plate 120 of the switching structure is arranged to result in a first flow path configuration as discussed above. A pressurizing means deliver a second mobile phase from a second mobile-phase source through mobile phase inlet conduit 48B, conduit 17C, conduit 130C, and conduit 17D. In addition, a first mobile phase is conveyed from the first mobile-phase source through conduit 48A. As a result, the first mobile phase forms a contiguous body of fluid that flows, through conduit 48A, conduit 17A, conduit 126C, conduit 17B, conduit 55, conduit 17E, conduit 128C, conduit 17F and conduit 53A. The mobile phase emerging from conduit 53A may be collected and recycled. Thus, conduit 55 is filled with a plug of the first mobile phase.

[0066] By forming a second flow path configuration as discussed above, the conduit 55 is now positioned in the flow path of the mobile phase entering the switching structure through conduit 48B. That is, the second mobile phase is now pumped through a flow path that travels, in order, through conduit 48B, conduit 17C, conduit 130C, conduit 17B, conduit 55, conduit 17E, conduit 128C and conduit 17D. Thus, the first mobile phase within conduit 55 is also forced through conduit 17D. It should be evident, then, that by rotating the substrate of the switching assembly, a first mobile-phase plug having a volume defined by conduit 55 is controllably introduced from a first mobile-phase source through conduit 17D followed by a second mobile phase. By providing fluid communication between conduit 17D and an inlet of a mobile-phase holding conduit (not shown), the mobile-phase holding conduit may be filled with a mobile phase that contains a first mobile-phase plug downstream from a second mobile-phase plug, thereby forming an overall mobile phase, contained in the mobile-phase holding conduit, that exhibits differing concentrations of a mobile phase component.

[0067] FIG. 5, illustrate a switching structure that employs rotational motion to controllably introduce plug of a first mobile phase, followed by, in order, a plug of a second mobile phase and the first mobile phase through a conduit. Like the switching structure illustrated in FIG. 4, the switching structure illustrated in FIG. 5 also employs rotational motion to controllably introduce a plug of a mobile phase. The switching structure 10 includes a substrate 12 and a switching plate 120 each having contact surface in slidable and fluid-tight relationship with each other. Eight conduits indicated at 17A, 17B, 17C, 17D, 17E, 17F, 17G and 17H, extend through the substrate and represent the vertices of an equilateral octagon.

[0068] The contact surface of the switching plate has located thereon four curved fluid-transporting channels. The four fluid-transporting channels lie along a circle having a diameter equal to the distance between two substrate conduits located farthest from each other. As a result, the fluid-transporting channels, in combination with substrate contact surface, form four conduits, 126C, 128C, 130C and 132C, as shown in FIG. 5.

[0069] Furthermore, two microconduits, indicated at 134 and 136, are provided as plug-holding chambers. Microconduit 134 provides fluid communication between substrate conduits 17B and 17D, and microconduit 136 provides fluid communication between substrate conduits 17E and 17H. Such microconduits may comprise, for example, commercially available capillary tubing. Depending on the relative orientation of the switching plate and the substrate, at least two possible flow paths configurations can be created. As shown in FIG. 5A, the first flow path configuration allows a first mobile phase from a first mobile-phase source (not shown) to travel, in order, through conduit 17A, conduit 126C, conduit 17B, conduit 134, conduit 17D, conduit 128C and conduit 17C. The first flow path configuration also allows a second mobile phase from a second mobile-phase source (not shown) to travel, in order, through conduit 17G, conduit 132C, conduit 17H, conduit 136, conduit 17E, conduit 130C, and conduit 17F. This configuration allows plug-holding chamber 134 to be filled by the first mobile phase and plug holding chamber 136 to be filled by the second mobile. By rotating the switching plate 120 45° about its center, a second flow path configuration results, as shown in FIG. 5B. The second flow path configuration forms a first flow path extending, in order, through conduit 17A, conduit 126C, conduit 17H, conduit 136, and conduit 17E, conduit 130C, conduit 17D, conduit 134, conduit 17B, conduit 128C and conduit 17C. The second flow path configuration also forms a second flow path extending, in order, through conduit 17G, conduit 132C, and conduit 17F. Thus, by rotating the substrate of the switching assembly, a first mobile-phase plug defined by conduit 134 is introduced through conduit 17C followed by a second mobile-phase plug defined by conduit 136, which is followed by additional first mobile phase. By providing fluid

communication between conduit 17C and an inlet of a mobile-phase holding conduit (not shown), the mobile-phase holding conduit may be filled with a mobile phase that contains a first mobile-phase plug downstream from a second mobile-phase plug, which is downstream from an additional first mobile-phase plug, thereby forming an overall mobile phase, contained in the mobile-phase holding conduit, that exhibits differing concentrations of a mobile phase component.

[0070] Thus, the invention provides, in yet another embodiment, a method for producing a flow of mobile phase. A mobile-phase source is provided comprising a mobile-phase-holding microconduit having a length defined by an upstream terminus and a downstream terminus, and an outlet port located at the downstream terminus, and a mobile phase, contained in the mobile-phase holding microconduit, that exhibits differing concentrations of selected mobile-phase component along the length of the microconduit a substrate. The mobile-phase holding microconduit is pressurized to force the mobile phase within the mobile-phase holding microconduit to flow toward the downstream terminus along the length of the microconduit and out of the outlet port. Optionally, at least one inlet port is provided fluid communication with the mobile-phase holding microconduit, wherein the outlet port is located downstream from the at least one inlet port of the mobile-phase holding microconduit. In such a case, a plurality of mobile-phase sources may be provided as well, each containing a mobile phase, wherein each mobile phase contains a different concentration of a selected mobile-phase component. Plugs of mobile phase from the mobile-phase sources are introduced through the at least one inlet port into the mobile-phase holding microconduit such that the plugs are arranged in a predetermined order along the length of the mobile-phase holding conduit.

[0071] In any of the above embodiments, suitable materials for forming the substrates and cover plates are selected with regard to physical and chemical characteristics that are desirable for proper functioning of the microfluidic device. In all cases, the substrate must be fabricated from a material that enables formation of high definition (or high "resolution") features, i.e., microchannels, chambers, and the like, that are of micrometer or submicrometer dimensions. That is, the material must be capable of microfabrication using, e.g., dry etching, wet etching, laser etching, laser ablation, molding, embossing, or the like, so as to have desired miniature surface features; preferably, the substrate is capable of being microfabricated in such a manner as to form features in, on, and/or through the surface of the substrate. Microstructures can also be formed on the surface of a substrate by adding material thereto. For example, polymer channels can be formed on the surface of a glass substrate using photo-imageable polyimide. In addition, a plurality of pieces or layers may be assembled to result in the formation of a substrate having a feature in, on, and/or through the surface of the substrate. Thus, for

example, a substrate having a channel located on a surface thereof may be formed from two layers, a first solid layer affixed to a second layer having a through-hole that in combination with the first solid layer form the channel. Also, all device materials that are used should be chemically inert and physically stable (e.g., in terms of pH, electric fields, etc.) with respect to any substance with which they come into contact when used to introduce a fluid sample. Suitable materials for forming the present devices include, but are not limited to, polymeric materials, ceramics (including aluminum oxide and the like), glass, metals, composites, and laminates thereof.

[0072] Polymeric materials are particularly preferred herein, and will typically be organic polymers that are either homopolymers or copolymers, whether naturally occurring or synthetic, and crosslinked or uncrosslinked. Specific polymers of interest include, but are not limited to, polyimides, polycarbonates, polyesters, polyamides, polyethers, polyurethanes, polyfluorocarbons, polystyrenes, poly(acrylonitrile-butadiene-styrene)(ABS), acrylate and acrylic acid polymers such as polymethyl methacrylate, other substituted and unsubstituted polyolefins, and copolymers thereof. Generally, at least one of the substrate and cover plate comprises a biofouling-resistant polymer when the microfluidic device is employed to transport biological fluids. Polyimide is of particular interest and has proven to be a highly desirable substrate material in a number of contexts. Polyimides are commercially available, e.g., under the tradename Kapton®, (DuPont, Wilmington, DE) and Upilex® (Ube Industries, Ltd., Japan). Polyetheretherketones (PEEK) also exhibit desirable biofouling-resistant properties.

[0073] The devices of the invention may also be fabricated from a "composite," i.e., a composition comprised of unlike materials. The composite may be a block composite, e.g., an A-B-A block composite, an A-B-C block composite, or the like. Alternatively, the composite may be a heterogeneous combination of materials, i.e., in which the materials are distinct separate phases; or it may be a homogeneous combination of unlike materials. As used herein, the term "composite" is used to include a "laminate" composite. A "laminate" refers to a composite material formed from several different bonded layers of identical or different materials. Other preferred composite substrates include polymer laminates, polymer-metal laminates (e.g., polymer coated with copper), ceramic-in-metal composites, or polymer-in-metal composites. One preferred composite material is a polyimide laminate formed from a first layer of polyimide, such as Kapton®, that has been co-extruded with a second, thin layer of a thermal adhesive form of polyimide known as KJ®, also available from DuPont (Wilmington, Delaware).

[0074] The present microfluidic devices can be fabricated using any convenient method, including, but not limited to, micromolding and casting techniques, embossing methods, surface micromachining, and bulk-

micromachining. The latter technique involves formation of microstructures by etching directly into a bulk material, typically using wet chemical etching or reactive ion etching ("RIE"). Surface micromachining involves fabrication from films deposited on the surface of a substrate. An exemplary surface micromachining process is known as "LIGA." See, for example, Becker et al. (1986), "Fabrication of Microstructures with High Aspect Ratios and Great Structural Heights by Synchrotron Radiation Lithography Galvanoforming and Plastic Moulding (LIGA Process)," *Microelectronic Engineering* 4(1): 35-36; Ehrfeld et al. (1988), "1988 LIGA Process: Sensor Construction Techniques via X-Ray Lithography," *Tech. Digest from IEEE Solid-State Sensor and Actuator Workshop*, Hilton Head, SC; Guckel et al. (1991) *J. Micromech. Microeng.* 1: 135-138. LIGA involves deposition of a relatively thick layer of an X-ray resist on a substrate followed by exposure to high-energy X-ray radiation through an X-ray mask, and removal of the irradiated resist portions using a chemical developer. The LIGA mold so provided can be used to prepare structures having horizontal dimensions--i.e., diameters--on the order of micrometers.

[0075] Another technique for preparing the present microfluidic devices is laser ablation. In laser ablation, short pulses of intense ultraviolet light are absorbed in a thin surface layer of material. Preferred pulse energies are greater than about 100 millijoules per square centimeter, and preferred pulse durations are shorter than about 1 microsecond. Under these conditions, the intense ultraviolet light photo-dissociates the chemical bonds in the substrate surface. The absorbed ultraviolet energy is concentrated in such a small volume of material that it rapidly heats the dissociated fragments and ejects them away from the substrate surface. Because these processes occur so quickly, there is no time for heat to propagate to the surrounding material. As a result, the surrounding region is not melted or otherwise damaged, and the perimeter of ablated features can replicate the shape of the incident optical beam with precision on the scale of about one micrometer or less. Laser ablation will typically involve use of a high-energy photon laser, such as an excimer laser of the F₂, ArF, KrCl, KrF, or XeCl type. However, other ultraviolet light sources with substantially the same optical wavelengths and energy densities may be used as well. Laser ablation techniques are described, for example, by Znotins et al. (1987) *Laser Focus Electro Optics*, at pp. 54-70, and in U.S. Patent Nos. 5,291,226 and 5,305,015 to Schantz et al.

[0076] The fabrication technique that is used must provide for features of sufficiently high definition, i.e., microscale components, channels, chambers, etc., such that precise "microalignment" of these features is possible, i.e., the features must be capable of precise and accurate alignment, including, for example, the alignment of complementary microchannels with each other, the alignment of projections and mating depressions,

the alignment of grooves and mating ridges, and the like. [0077] For any of the inventive devices, the fluid-transporting features may be formed, independently or otherwise, through laser ablation or other techniques discussed below or known in the art. It will be readily appreciated that, although the microchannels have been represented in a generally extended form, microchannels for this and other embodiments can have a variety of configurations, such as a straight, serpentine, spiral, or any tortuous path. Further, the microchannels can be formed in a wide variety of crosssectional channel geometries, including semi-circular, rectangular, rhomboidal, and the like; and the channels can be formed in a wide range of aspect ratios.

[0078] In some instances, the substrate and the cover plate may be formed in a single, solid flexible piece. Microfluidic devices having a single-piece substrate and cover plate configuration have been described, e.g., in U.S. Patent Nos. 5,658,413 and 5,882,571, each to Kaltenbach et al. However, the cover plate and substrate of the inventive device are typically formed as discrete components. In such a case, microalignment means described herein or known to one of ordinary skill in the art may be employed to align the cover plate with the substrate. To ensure that the conduit or conduits formed between the substrate and the cover plate are fluid-tight, pressure-sealing techniques may be employed, e.g., by using external means (such as clips, tension springs, or an associated clamp), internal means (such as male and female couplings), or chemical means (e.g., adhesive or welding) to hold the pieces together. As with all embodiments described herein, the pressure-sealing techniques may allow the contact surfaces to remain in fluid-tight contact under an internal microfluidic device fluid pressure of up to about 100 megapascals, typically about 0.5 megapascals to about 40 megapascals.

[0079] The separation conduit for the inventive device is constructed for separation as generally described in U.S. Serial No. 09/908,231. Aspects of known separation technology may be incorporated in the practice of the present invention. For example, the microfluidic device may further comprise separation media within a separation conduit, or a polymeric material formed *in situ* within the separation conduit. When ordinary liquid chromatography packing material is slurry-packed within the separation conduit, a frit structure, micromachined or otherwise, may be included near or at the sample outlet port. The frit structure serves to ensure that the packing material is not displaced from within the sample conduit when a fluid sample and/or a mobile phase are conveyed through the conduit. In addition, it is preferred that the cross-sectional area of the separation conduit be reduced downstream from the frit structure, particularly if the sample outlet port is a part of an electrospray tip as described, for example, in U.S. Serial No. 09/711,804 ("A Microfluidic device Having an Integrated Protruding Electrospray Emitter and a Method for Producing the Mi-

crofluidic device"), inventors Brennen, Yin, and Killeen, filed on November 13, 2000. Alternatively, the separation conduit may exhibit a high surface area-to-volume ratio.

5 [0080] Thus, the conduit may exhibit any micromachined structure appropriate for liquid chromatography. In addition, or in the alternative, the conduit may contain any of a number of known liquid chromatographic packing materials. Such packing materials typically exhibit a 10 surface area of about 100 m²/g to about 500 m²/g. The conduit may be adapted to separate fluid sample components according to molecular weight, polarity, hydrophobicity, or other properties through techniques known to one of ordinary skill in the art (e.g., through proper 15 selection of packing materials).

[0081] Similarly, an analyzer may be interfaced with any portion of the flow path of the inventive microfluidic device, including the inlet port. The analyzer may be, for 20 example, a mass spectrometer, in which case the outlet port could be located within or adapted to deliver fluid sample to an ionization chamber. See U.S. Serial No. 09/711,804 ("A Microfluidic device Having an Integrated Protruding Electrospray Emitter and a Method for Producing the Microfluidic device"), inventors Brennen, Yin, and Killeen, filed on November 13, 2000. In addition, mass spectrometry technologies are well known in the 25 art and may involve, for example, laser desorption and ionization technologies, for which uses in conjunction with microfluidic devices are described in U.S. Patent Nos. 5,705,813 and 5,716,825. In the alternative or in 30 addition, the analyzer may be a source of electromagnetic radiation configured to generate electromagnetic radiation of a predetermined wavelength. Depending on the intrinsic properties of the fluid sample and/or any 35 molecular labels used, the radiation may be ultraviolet, visible, or infrared radiation.

[0082] From the above description of the various 40 embodiments of the invention, it is evident that a sample-introducing means appropriate to the desired separation process and the dimensions of the microfluidic device may be used to introduce a predetermined volume of 45 fluid sample. Typically, the predetermined volume is less than about 5 µL. Preferably, the predetermined volume is about 0.005 µL to about 1 µL. Optimally, the predetermined volume is about 0.01 µL to about 0.1 µL. Examples of sample-introducing means are described in 50 U.S. Serial Nos. 09/908,231 and 09/908,292. Additional sample-introducing means include, but are not limited to, syringes, micropipettes, inkjet printheads, sippers, and other devices known in the art.

[0083] Variations of the invention, not explicitly disclosed herein, will be apparent to those of ordinary skill in the art. Other features may be included to carry out known reactions and processes, for example, reactions and processes associated with sample preparation, synthesis, and analysis. Such features may be formed from conduits and channels that provide for fluid flow in a parallel or a nonparallel direction with respect to the 55

contact surfaces. Moreover, cascades may be formed as channels on a single substrate surface, as features on a switching mechanism, or as an assembly of conduits formed as a distinct and separate from the separation column. In addition, one of ordinary skill may adapt the structures and the components of structures discussed above to operate in combination, optionally with other microfluidic flow control mechanisms. In addition to the use of diffusion to create a gradient, micro-mixers known the art may be incorporated to accelerate mixing.

[0084] It is to be understood that, while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

[0085] All patents, patent applications, and publications mentioned herein are hereby incorporated by reference in their entireties.

Claims

1. A microfluidic device (10) for separating the components of a fluid sample, the microfluidic device comprising:

a substrate (12) having a microchannel (18) formed in a surface (14) thereof;
a cover plate (40) arranged over the substrate surface (14), the cover plate (40) in combination with the microchannel (18) defining a separation conduit (19) for separating the components of the fluid sample according to a specific component property, wherein the separation conduit (19) has an inlet port (72) and an outlet port (47); and
an integrated gradient-generation means for generating a gradient of a selected mobile-phase component in a mobile phase, and adapted to allow the mobile phase from the gradient-generation means to be transported through the inlet port (72) into the separation conduit (19) and out of the outlet port (47).

2. A microfluidic device (10) for separating the components of a fluid sample, the microfluidic device (10) comprising:

a substrate (12) having a microchannel (18) formed in a surface (14) thereof;
a cover plate (40) arranged over the substrate surface (14), such that the cover plate (40), in combination with the microchannel (18), defines a separation conduit (19) for separating the components of the fluid sample according

to a specific component property, wherein the separation conduit (19) has an inlet port (72) and an outlet port (47); and
an integrated mobile-phase source comprising a microconduit (21) having a length defined by an upstream terminus and a downstream terminus, the microconduit (21) containing a mobile phase that exhibits a gradient of a selected mobile-phase component along the length of the microconduit,

wherein the integrated mobile-phase source is arranged to allow the mobile phase to be transported through the inlet port (72) into the separation conduit (19) and out of the outlet port (47).

3. A microfluidic device (10) for separating the components of a fluid sample, the microfluidic device (10) comprising:

(a) a gradient-generation means for generating a gradient of a selected mobile-phase component in a mobile phase comprising

(i) a substrate (12) having a microchannel formed in a surface (14) thereof, wherein the microchannel has an upstream terminus and a downstream terminus,
(ii) a cover plate (40) arranged over the substrate surface (14), wherein the cover plate, in combination with the microchannel, forms a mobile-phase holding conduit (21) having a length defined by the upstream terminus and the downstream terminus,
(iii) a plurality of inlet ports (48) arranged along the length of the mobile-phase holding conduit, and
(iv) an outlet port (52) located downstream from the inlet ports (48) of the mobile-phase holding conduit;

(b) a separation conduit (19) for separating the components of a fluid sample according to a specific component property; and

(c) a means for introducing the mobile phase from the gradient-generation means into the separation conduit (19).

4. The microfluidic device (10) of any preceding claim, further comprising separation media within the separation conduit.
5. The microfluidic device (10) of any preceding claim, further comprising a polymeric material formed *in situ* within the separation conduit.
6. The microfluidic device (10) of any preceding claim,

- wherein the separation conduit exhibits a high surface area-to-volume ratio.
7. The microfluidic device (10) of any preceding claim, wherein the component property is selected from the group consisting of molecular weight, polarity, hydrophobicity, and charge. 5
8. The microfluidic device (10) of claim 1, further comprising two mobile-phase sources in fluid communication with the integrated gradient-generation means, 10 wherein at least one of the mobile-phase sources contains the selected mobile-phase component.
9. A microfluidic device (10) for producing a flow of mobile phase, comprising: 15
- (a) mobile-phase source comprising
 - (i) a mobile-phase holding microconduit (21) having a length defined by an upstream terminus and a downstream terminus, and an outlet port (52) located at the downstream terminus, and
 - (ii) a mobile phase, contained in the mobile-phase holding microconduit (21), that exhibits differing concentrations of selected mobile-phase component along the length of the mobile-phase holding microconduit; and
 - (b) a means for pressurizing the microconduit to force the mobile phase within the microconduit (21) to flow toward the downstream terminus along the length of the microconduit (21) and out the outlet port (52). 30
10. A microfluidic device (10) for producing a flow of mobile phase, comprising: 40
- (a) a means for producing different concentrations of a selected mobile-phase component in different locations within a mobile phase comprising
 - (i) a mobile-phase-holding microconduit (21) having a length defined by an upstream terminus and a downstream terminus,
 - (ii) an outlet port (52) located at the downstream terminus of the mobile-phase holding microconduit (21), and
 - (iii) at least one inlet port (48) in fluid communication with the mobile-phase holding microconduit (21) upstream from the outlet port (52); 45
 - (b) a plurality of mobile-phase sources each containing a mobile phase, wherein each mobile phase contains a different concentration of a selected mobile-phase component;
 - (c) a means for introducing plugs of mobile phase from the mobile-phase sources through the at least one inlet port into the mobile-phase holding conduit (21) such that the plugs are arranged in a predetermined order along the length of the mobile-phase holding conduit (21); and
 - (d) a means for pressurizing the microconduit to force the mobile phase within the microconduit (21) to flow toward the downstream terminus along the length of the microconduit (21) and out the single outlet port (52). 50
11. The microfluidic device of either claim 9 or claim 10, further comprising a separation conduit (19) in fluid communication with the outlet port of the microconduit (52). 55
12. A method for separating the components of a fluid sample, comprising:
- (a) providing a microfluidic device as claimed in any preceding claim;
 - (b) using the integrated gradient-generation means to generate a gradient of the selected mobile-phase component in the mobile phase,
 - (c) controllably introducing a predetermined volume of the fluid sample from a sample source into the separation conduit; and
 - (d) conveying the fluid sample through the separation conduit using a mobile phase, thereby separating the components of the fluid sample. 60
13. The method of claim 12, further comprising, during or after step (d), (e) analyzing the fluid sample flowing in the separation conduit or from the outlet port of the separation conduit. 65
14. The method of claim 12 or 13, wherein step (b) further comprises (b') transporting components of the mobile phase into the integrated gradient-generation means. 70
15. The method of claim 14, wherein step (b) further comprises, after step (b'), allowing a sufficient amount of time to pass to result in diffusion of the components of the mobile phase to form a non-stepwise gradient in the mobile phase. 75
16. The method of any of claims 12 to 16, wherein step (d) is carried out using mobile phase flow at a rate of no more than about 1 μ L/min. 80

17. A method for separating the components of a fluid sample, comprising:

- (a) using no more than about 100 μL of fluid to produce a mobile phase containing a gradient of the selected mobile-phase component; and
(b) conveying a fluid sample through the separation conduit using the mobile phase containing a gradient of the selected mobile-phase component, thereby separating the components of the fluid sample.

18. A method for producing a flow of mobile phase, comprising:

- (a) providing a mobile-phase source comprising

- (i) a mobile-phase-holding microconduit having a length defined by an upstream terminus and a downstream terminus, and an outlet port located at the downstream terminus, and
(ii) a mobile phase, contained in the mobile-phase holding microconduit, that exhibits differing concentrations of selected mobile-phase component along the length of the microconduit a substrate; and

- (b) pressurizing the microconduit to force the mobile phase within the mobile-phase holding microconduit to flow toward the downstream terminus along the length of the microconduit and out of the outlet port.

19. The method of claim 18, wherein the mobile-phase holding microconduit is further defined by a substrate having a microchannel formed in a surface thereof in combination with a cover plate arranged over the substrate surface.

20. The method of claim 18 or 19, wherein step (a) comprises:

- (a') providing at least one inlet port in fluid communication with the mobile-phase holding microconduit, wherein the outlet port is located downstream from the at least one inlet port of the mobile-phase holding microconduit;
(a'') providing a plurality of mobile-phase sources each containing a mobile phase, wherein each mobile phase contains a different concentration of a selected mobile-phase component; and
(a''') introducing plugs of mobile phase from the mobile-phase sources through the at least one inlet port into the mobile-phase holding microconduit such that the plugs are arranged in a

predetermined order along the length of the mobile-phase holding conduit, the predetermined order.

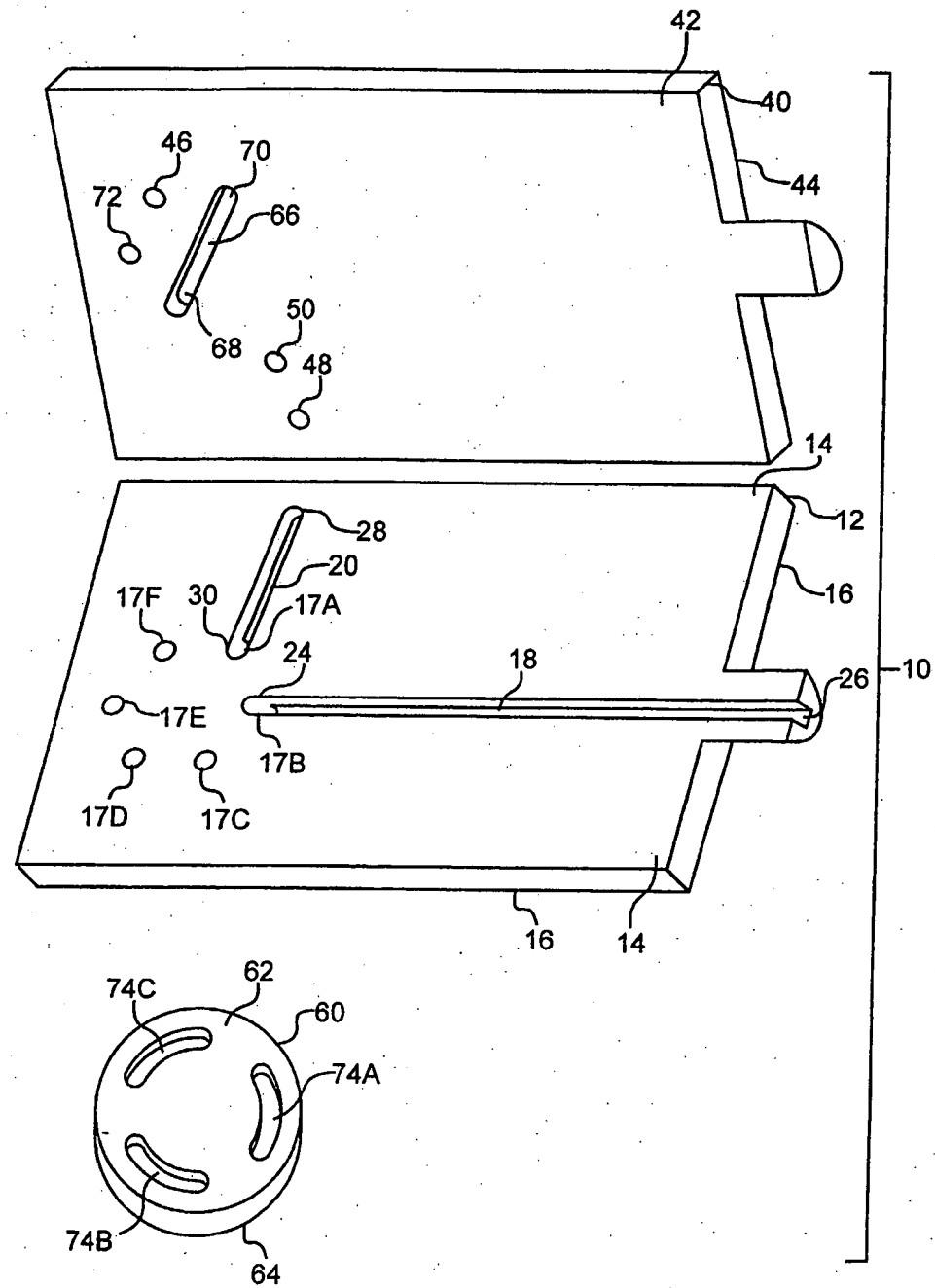


FIG. 1A

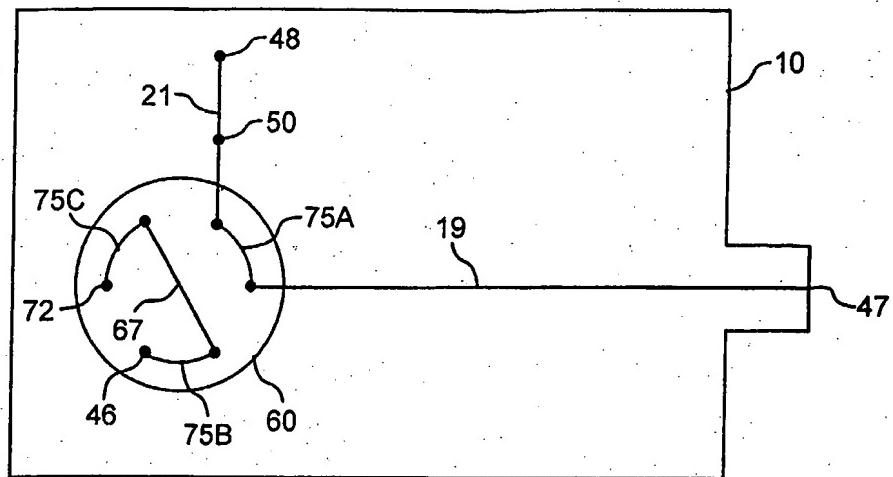


FIG. 1B

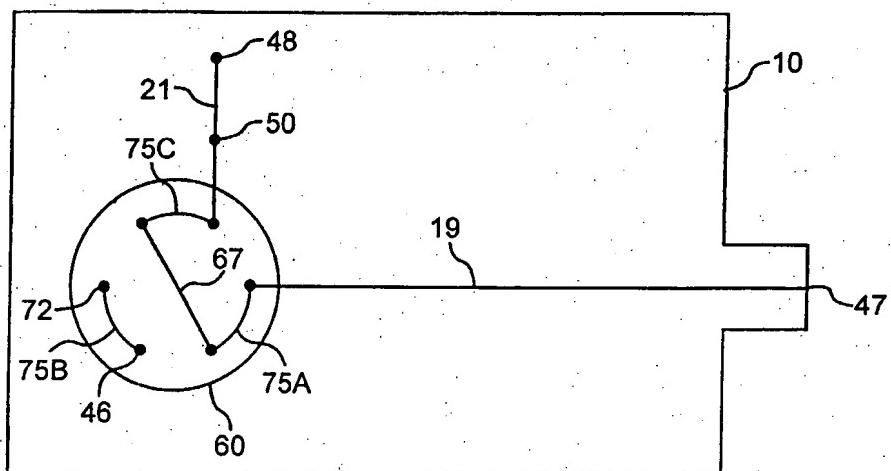


FIG. 1C

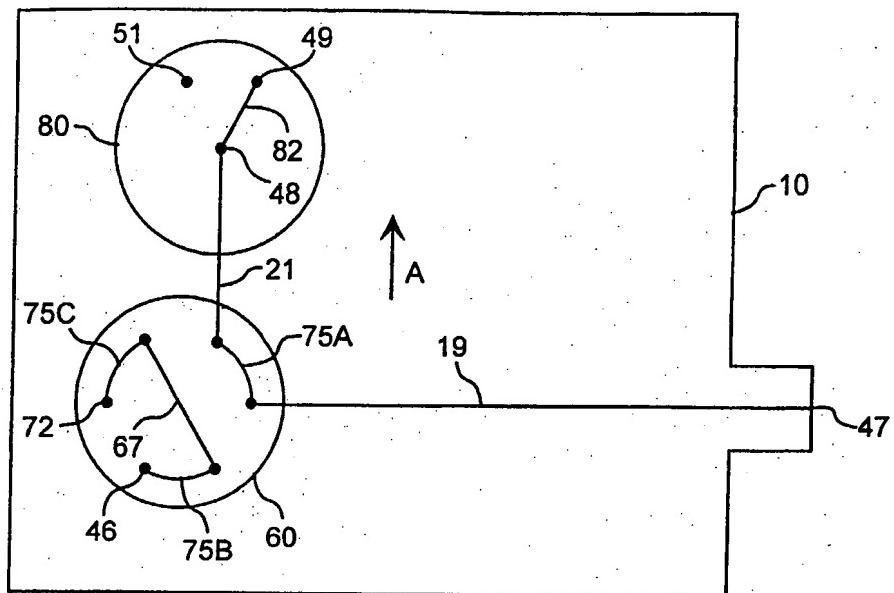


FIG. 2A

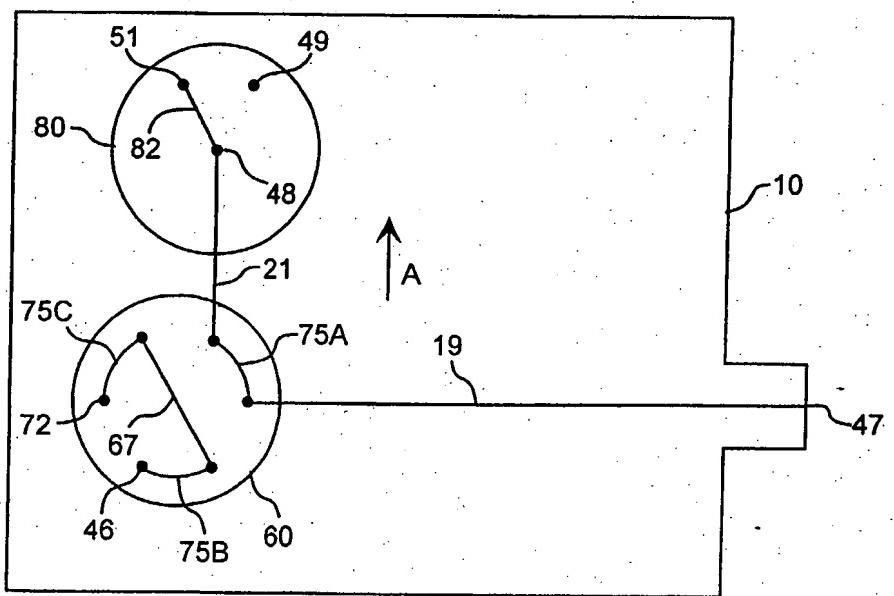


FIG. 2B

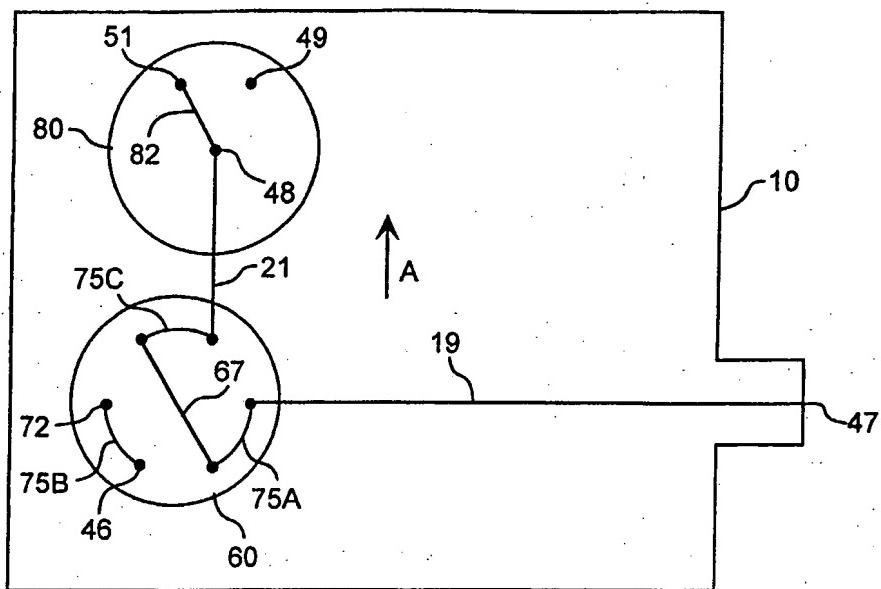


FIG. 2C

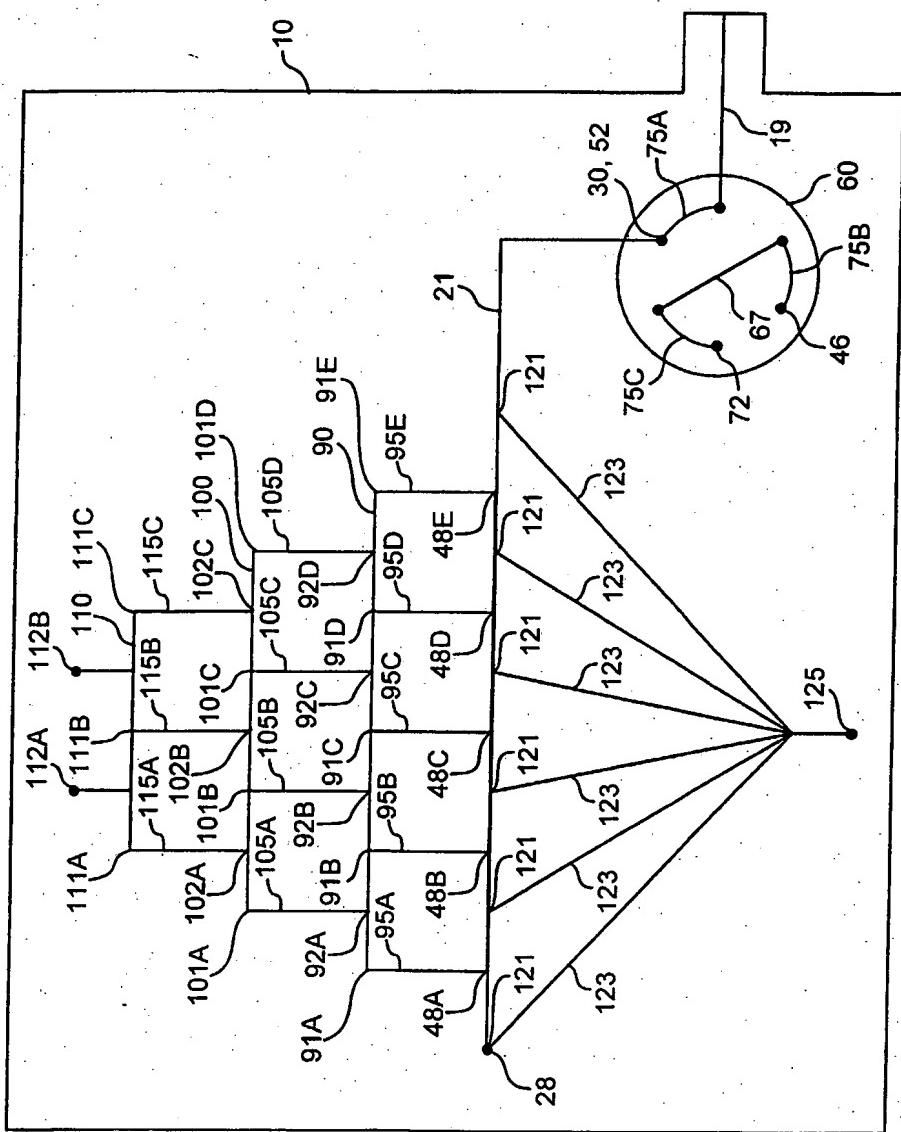


FIG. 3

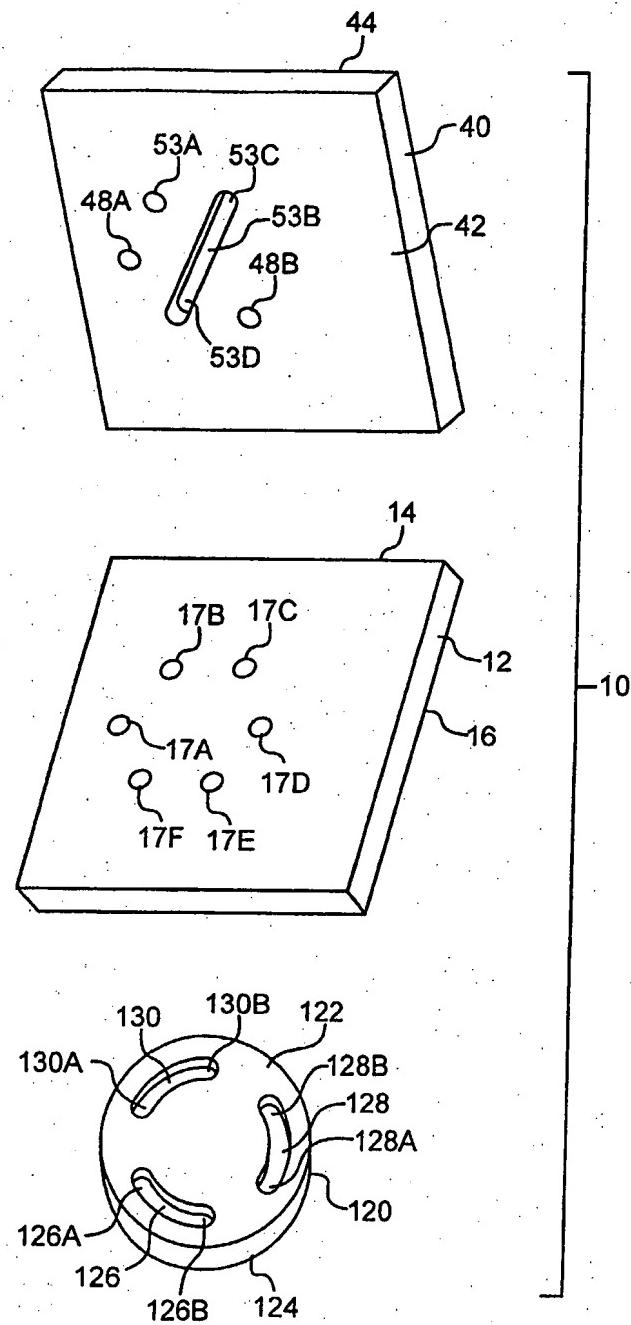


FIG. 4A

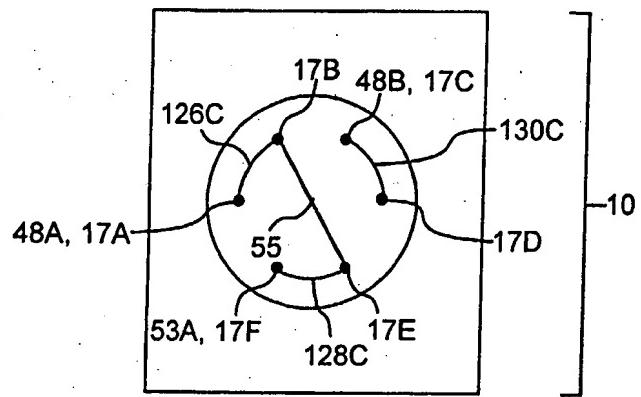


FIG. 4B

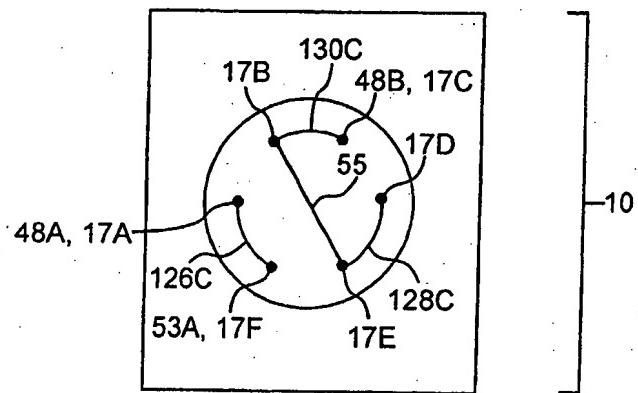


FIG. 4C

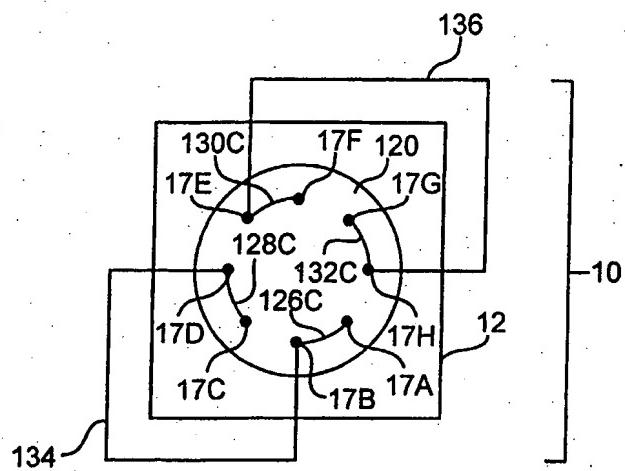


FIG. 5A

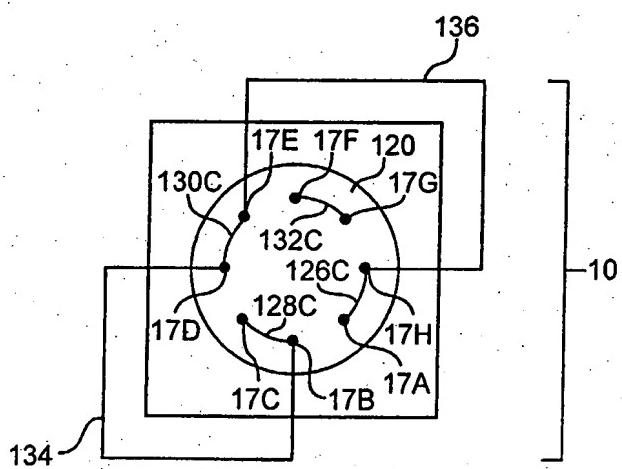


FIG. 5B



(12)

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PROCEDE DESTINES A LA DETECTION QUALITATIVE ET/OU QUANTITATIVE DE CELLULES

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| US-B1- 6 187 546 | |

- **HARDINGHAM J E ET AL: "IMMUNOBED-PCR: A TECHNIQUE FOR THE DETECTION OF CIRCULATING TUMOR CELLS USING IMMUNOMAGNETIC BEADS AND THE POLYMERASE CHAIN REACTION" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, Bd. 53, 1. August 1993 (1993-08-01), Seiten 3455-3458, XP000605489 ISSN: 0008-5472**
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Bemerkungen:

Die Akte enthält technische Angaben, die nach dem Eingang der Anmeldung eingereicht wurden und die nicht in dieser Patentschrift enthalten sind.

Beschreibung

[0001] Die vorliegende Erfindung betrifft ein Verfahren zum qualitativen und/oder quantitativen Nachweis von Zellen in einer Probe. Derartige Nachweisverfahren werden insbesondere bei der Diagnostik oder Behandlungskontrolle von Tumorerkrankungen benötigt. Denn im Rahmen der Krebsvor- oder -nachsorge ist es von großer Wichtigkeit maligne Tumore bzw. rezidive maligne Tumore anhand des Auftretens metastasierender Tumorzellen im Blut frühzeitig nachweisen zu können. Das vorliegende Verfahren wird jedoch nicht nur hier eingesetzt sondern kann ganz grundsätzlich zum Nachweis und zur Erkennung von seltenen Zellen in biologische Zellen enthaltenden Proben eingesetzt werden. Dies kann beispielsweise auch zum Nachweis von fötalen Zellen in maternalem Blut oder auch zum Nachweis von Stammzellen erfolgen.

[0002] Hodenkrebs ist für weniger als 2% aller bösartigen Neubildungen von Tumoren beim Mann verantwortlich. Allerdings handelt es sich bei 20-30% aller Krebserkrankungen unter 40jähriger Männer um Hodenkrebs. Die Zahl der jährlichen Neuerkrankungen beispielsweise in der Bundesrepublik Deutschland beträgt ca. 3600, wobei ca. 240 Männer an Hodenkrebs sterben. Die höchste Inzidenz findet man dabei zwischen dem 25. und 40. Lebensjahr. Durch den Fortschritt in der onkologischen Therapie können heute über 90% aller Betroffenen langfristig geheilt werden. Die hohen Überlebensraten begründen sich dabei in der ausgeprägten Wirksamkeit der cis-Platin-basierenden Chemo-therapien.

[0003] Brustkrebs ist die häufigste Diagnose, wenn eine Tumorerkrankung bei Frauen festgestellt wird (26,4% aller Neuerkrankungen). Trotz massiver Bemühungen, die in Früherkennung, Behandlung und Nachsorge aufgewendet werden, rangiert diese Erkrankung immer noch an erster Stelle krebsbedingter Todesursachen bei der Frau. Die Erkrankungszahlen in den westlichen Industrieländern nehmen in den vergangenen Jahren trotz verstärkter Bemühungen um die Früherkennung weiter zu. Problematisch ist die hohe Metastasierungsrate nach Erstbehandlung, die in der Mehrzahl der Fälle bereits nach 1-3 Jahren zum Tod der Patientin führt. Hauptgrund hierfür ist die Streuung von Tumorzellen in frühen Stadien der Tumorentwicklung. Neben der Ersterkennung eines Mammakarzinoms ist daher insbesondere der frühestmögliche Nachweis metastasierender Zellen für eine erfolgreiche Behandlung von entscheidender Bedeutung. Ebenso kann im klinischen Stadium I ein definitiver Negativnachweis hilfreich sein, wenn zu entscheiden ist, ob die Patientin mit einer Chemotherapie oder einer Operation belastet werden muss.

[0004] Beim kolorektalen Primärtumor ist die Tumorprogression nach der Resektion in erster Linie auf residuale Tumorzellen zurückzuführen. Diese Zellen werden prä- oder intraoperativ aus dem primären Tumor abgelöst und erhalten die Möglichkeit zur Verstreutung im ganzen Organismus.

[0005] Neben der Ersterkennung eines kolorektalen Karzinoms ist daher insbesondere der frühestmögliche Nachweis metastasierender Zellen für eine erfolgreiche Behandlung von entscheidender Bedeutung.

[0006] Dabei gilt es, im klinischen Stadium 1 der Erkrankungen zu entscheiden, ob der Patient mit einer Chemotherapie und/oder mit einer Operation belastet werden muss, um einen dauerhaften Heilungserfolg zu erzielen. Eine große Anzahl von Patienten wird dabei chemotherapeutisch behandelt, obwohl kein gesicherter Nachweis einer Metastasierung vorliegt. In den Konzepten, die auf einer reinen Überwachung aufbauen, kommt es jedoch in 25% der Fälle zu Rezidiven mit zum Teil tödlichem Ausgang.

[0007] Bei den derzeit angewandten Untersuchungsmethoden werden bei Krebspatienten sogenannte Tumormarker auf Proteinebene (immunologisch bzw. enzymatisch) quantitativ im Blut oder in anderen Körperflüssigkeiten ermittelt. Diese Nachweisverfahren sind jedoch für die Tumordiagnostik bzw. Behandlungskontrolle/Nachsorge bei Tumoren nur bedingt geeignet, da erhöhte Tumormarkerwerte in Körperflüssigkeiten auch durch nichttumoröse Erkrankungen, wie beispielsweise Entzündungen des Magen-Darm-Traktes, Leberzirrhose, Virusinfekte oder starkes Rauchen hervorgerufen werden können.

[0008] Molekulargenetische Verfahren erscheinen hier für den Nachweis von Tumorzellen im peripheren Blut hilfreich, da am Beginn des Metastasierungsprozesses der Übertritt von Tumorzellen ins venöse Blut stehen kann.

[0009] Die EP 0 520 794 B1 offenbart ein derartiges Verfahren, bei dem Metastasierungen in Körperfgeweben oder Flüssigkeiten erfasst werden. Dabei werden Nukleinsäuren erfasst, beispielsweise mittels Vervielfältigung durch Polymerase-Kettenreaktion. Das Verfahren beruht nun entscheidend darauf, dass die nachgewiesene Nukleinsäuresequenz in Zellen des Herkunftsgewebes eines Tumors exprimiert wird, d.h. in Tumorzellen und markerabhängig auch in den gesunden Zellen des Herkunftsgewebes. Weitere Bedingung ist, dass diese Sequenz normalerweise nicht in denjenigen Zellen exprimiert wird, deren Gewebe untersucht wird. Wird also eine entsprechende Sequenz in der untersuchten Probe gefunden, so muss diese von verschleppten, d.h. metastasierenden Zellen eines ortsfremden Tumors herrühren. Damit beruht dieses Verfahren letztlich auf dem Nachweis von Zellen, die in der Blutprobe von gesunden Personen nicht vorkommen sollten.

[0010] Insgesamt ist festzustellen, dass die derzeit verwendeten diagnostischen Methoden zu ungenau sind, wenn es um die Beurteilung der malignen Potenz von Resttumoren nach durchgeföhrter Chemotherapie in den metastasierenden Stadien geht. Es gilt also weiterhin, Nachweise für eine okkluse bzw. restliche Metastasierung zu finden, die eine rechtzeitige Einordnung in die vielfältigen primär kurativen therapeutischen Optionen zulassen. Wesentliches

Problem ist es hierbei, dass die zu erfassenden Zellen, beispielsweise Tumorzellen im peripheren Blut, nur in extrem geringer Zahl vorkommen.

[0011] Die WO 98/12227 A1 offenbart ein Verfahren zum Nachweis der Expression des GP-54-Antigens. Hierzu werden zuerst aus einer Probe Zellen immunologisch mittels zweier gegen verschiedene Epitope des GP-54-Antigens gerichtete Antikörper isoliert. Für diese isolierten Zellen wird anschließend der Nachweis der Expression von GP-54 durch Analyse der mRNA erbracht.

[0012] Die WO 97/37226 A1 offenbart ein Verfahren zur Gewinnung von Tumorzellen oder Fragmenten von Tumorzellen mittels eines Antikörpers bzw. eines Antikörpermischungssatzes. Die Analyse der so gewonnenen Zellen oder Zellfragmente erfolgt über optische Verfahren, die es dem Betrachter ermöglichen, Tumorzellen von normalen Zellen zu unterscheiden.

[0013] Die WO 96/29430 A1 offenbart die Erfassung von Tumorzellen mit einem Multimarkerassay. Hierbei werden zwei oder mehrere mRNA-Marker in Proben, beispielsweise Blutproben, untersucht. Die Resultate dieses molekularbiologischen Multimarker-Nachweises werden mit einer statistisch signifikanten Referenzgruppe von Normalpatienten und Melanom- oder Brustkrebspatienten verglichen und auf Basis dieses Vergleichs eine Korrelation der Anzahl und Art der Marker mit verschiedenen klinischen Zuständen durchgeführt. Ziel dieses Verfahrens ist es, okklute Tumoren auch bei Patienten zu erfassen, bei denen keine klinischen Anzeichen für einen Tumor vorhanden sind.

[0014] Hardingham et al.: "Immunobead-PCR: A technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction", Cancer Research, Band 53 (1993), Seiten 3455 bis 3458 offenbart ein Verfahren zur Erfassung von disseminierten Tumorzellen in Blutproben. Hierzu erfolgt zuerst ein immunologischer Anreicherungsschritt mittels eines Antikörpers gefolgt von dem Nachweis einer DNA-Mutation. Für den immunologischen Anreicherungsschritt wird aus einer Auswahl von Antikörpern experimentell der geeigneteste Antikörper ermittelt und anschließend für das Verfahren eingesetzt.

[0015] Im Kenntnis von diesem Stand der Technik ist es Aufgabe der vorliegenden Erfindung, ein Verfahren zur Verfügung zu stellen, mit dem auf einfache, sichere und wiederholbare Weise seltene biologische Zellen in einer biologischen Zellen enthaltenden Probe mit hoher Sensitivität und Spezifität nachgewiesen werden können.

[0016] Diese Aufgabe wird durch das Verfahren nach Anspruch 1 gelöst. Vorteilhafte Weiterbildungen des erfindungsgemäßen Verfahrens werden in den jeweiligen abhängigen Ansprüchen gegeben.

[0017] Entscheidend bei dem erfindungsgemäßen Verfahren ist es nun, dass zuerst die zu selektierenden bzw. nachzuweisenden Zellen mittels einer Kombination von Antikörpern oder Antikörperderivaten oder einem bispezifischen Antikörper oder Antikörperderivat markiert werden. Dadurch ist es möglich, insbesondere die gesuchten Zellen zu markieren, abzutrennen und damit anzureichern. Dies bedeutet, dass in einem ersten Schritt ein kombinierter immunologischer Nachweis bzw. Selektionierung erfolgt. Unter Antikörperderivat wird in dieser Anmeldung jede Art von verändertem Antikörper oder Antikörperfragment verstanden, das eine Bindungsstelle aufweist, beispielsweise einkettige Antikörper, Antikörperfragmente wie Fab-Fragmente oder rekombinante Antikörper. Im folgenden sind, wenn von "Antikörper" gesprochen wird, immer Antikörper und/oder Antikörperderivate bezeichnet.

[0018] In einem zweiten Schritt werden dann auf molekularbiologischer Basis mit einer vordefinierten Kombination von Nachweisreagentien mindestens ein Marker erfasst, der für die gesuchten Zellen spezifisch ist bzw. in diesen präferentiell zu finden ist, so dass hier wiederum spezifisch die gesuchten Zellen ausgewählt werden. Es handelt sich hier also um einen kombinierten molekularbiologischen Nachweis. Grundgedanke der vorliegenden Erfindung ist also, einen Nachweis über eine Kombination immunologischer Parameter mit einem Nachweis über eine Kombination molekularbiologischer Parameter zu kombinieren. Überraschenderweise ergeben sich hierdurch ganz hervorragende Nachweisergebnisse, mit denen in Detektionsbereiche vorgestoßen wird, die sämtlichen bekannten Techniken aus dem Stand der Technik bisher nicht zugänglich waren. So können Konzentrationen gesuchter Zellen in Blutproben bis herab zu zwei Zellen pro 5 Milliliter noch nachgewiesen werden. Eine derartige Spezifität und Sensitivität wird bisher im Stand der Technik nicht erreicht.

[0019] Eukaryontische Zellen tragen eine Vielzahl unterschiedlicher Moleküle an ihrer Zelloberfläche. Entsprechend des Ursprungs und der Funktion der einzelnen Zelle unterscheidet sich die Kombination der exprimierten Oberflächenmoleküle, so dass zelltypspezifische Muster entstehen. Zur Erkennung dieser zelltyp-spezifischen Muster werden Antikörper genutzt. Antikörper binden mit hoher Spezifität an ihr Antigen, hier an ausgewählte Oberflächenmoleküle. Diese Eigenschaft wird genutzt, um Zellen mittels spezifischer Antikörperbindung anhand ihrer Zelltyp-spezifischen Muster zu erkennen und voneinander zu unterscheiden.

[0020] So unterscheidet beispielsweise die Expression spezieller Oberflächenproteine von Tumorzellen von nicht-transformierten Zellen dieses Zelltyps.

[0021] Dem Nachweis der Marker geht eine Selektion der Zielzellen über die Bindung verschiedener Antikörper an die gesuchten Zellen voraus. Denn die Expression spezieller Oberflächenproteine unterscheidet Zellen eines Typs von Zellen eines anderen Typs. So unterscheidet beispielsweise die Expression spezieller Oberflächenproteine Tumorzellen von nicht-transformierten Zellen dieses Zelltyps.

[0022] Da sich das spezielle Muster der Oberflächen-Antigene beispielsweise bei Tumorzellen auch von den Blut-

zelltypischen Mustern unterscheidet, können Tumorzellen im Blut unterschieden werden. Um Tumorzellen zu identifizieren, werden Antikörper, die diese speziellen Oberflächenproteine spezifisch erkennen, als Werkzeuge genutzt. Die spezifische Antikörperbindung wird für verschiedene Analyse- und Separations-Methoden nutzbar gemacht.

[0023] Aufgrund der intensiven Bindung von speziell dafür selektierten Immunglobulinen ist neben der Erkennung von Zellen über deren Oberflächenepitope auch eine Separierung der erkannten Zellen von nicht erkannten möglich.

[0024] Verschiedene Trennprinzipien sind möglich:

1. Trennprinzip beruhend auf Flüssigphase; z.B. Durchflusszytometrie:

Für die durchflußzytometrische Analyse werden Antikörper mit Fluoreszenzfarbstoffen gekoppelt. Vereinzelte Zellen werden in einem konstanten Flüssigkeitsstrom einzeln an einer Lichtquelle (Laser) vorbeigeleitet. Bei Beleuchtung der Zellen werden die an den Antikörpern gebundenen Fluoreszenzfarbstoffe angeregt und strahlen Licht bestimmter Wellenlängen ab. Das abgestrahlte Licht wird detektiert und das gemessene Signal digitalisiert gespeichert. Das Lichtsignal kann einzelnen Zellen zugeordnet werden. Die Antikörper-markierte Zelle wird so erkannt und kann nun von anderen Zellen getrennt werden. Zur Trennung werden die Zellen in kleinsten Tropfen vereinzelt. Nach Erkennung der Antikörper-markierten Zelle wird der entsprechende Tropfen in ein Auffangbehältnis gelenkt. Eine derartige Anreicherung kann beispielsweise durch FACS-Durchflusszytometrie erfolgen. Dabei werden beispielsweise angereicherte Zellen mit fluoreszenzmarkierten monoklonalen Antikörpern gegen tumorspezifische Oberflächenproteine inkubiert. Die markierten Zellen werden zweifach mit PBS gewaschen und im Anschluss werden 10^7 Zellen in 1 ml PBS resuspendiert. Für die Isolierung der Tumorzellen wird ein FACS Vantage SE-Durchflusszytometer (Becton Dickinson) verwendet. Über das CellQuest Programm erfolgen Datenaufnahme, Instrumentenkontrolle und Datenauswertung. Die sortierten Zellen werden in ein 1,5 ml-Reaktionsgefäß (gefüllt mit 1 ml PBS) überführt. Die RNS kann dann wie später beschrieben isoliert werden.

2. Trennprinzip beruhend auf Festphase; z.B. magnetische Separation:

Für die magnetische Separation werden Antikörper an pseudomagnetische Partikel gekoppelt. Nach Einbringen der pseudomagnetischen Partikel in ein Magnetfeld wandern die Partikel im magnetischen Feld. Bei der Bewegung in diesem magnetischen Feld werden Zellen, an die diese gekoppelten Antikörper gebunden sind, mitgerissen und von anderen Zellen getrennt.

[0025] Zur Zellerkennung mittels Magnetpartikel werden folglich an pseudomagnetische Partikel, die eine definierte Anzahl an chemisch aktivierten Stellen auf ihrer Oberfläche besitzen, Antikörper gekoppelt. Kopplungsverfahren sind beispielsweise aus James P. Gosling, Solid-phase Concepts and Design, in: R.F. Masseyeff, W.H. Albert N.A. Staines (eds), Methods of Immunological Analysis, Vol. 1, VCH Verlagsgesellschaft mbH, Weinheim, pp. 507-529 bekannt. Über die Spezifität der Antikörper wird die Spezifität der Trennung bestimmt. Eine Ziel-Zellen enthaltende Blutprobe wird mit Antikörper-gekoppelten Magnetpartikeln versetzt; dann werden Partikel und Blut relativ zueinander bewegt, beispielsweise durch "Über-Kopf-Rotieren" in einem geschlossenen Behälter befindlicher Proben oder durch Bewegung der Partikel aufgrund wechselnder Magnetfelder. Jene Ziel-Zellen, die von einem der Festphase-gebundenen Antikörper erkannt und damit fest gebunden werden, folgen der Bewegung der Partikel. Hierdurch ist es möglich, bei Anlegen eines magnetischen Feldes, die Partikel mit den daran gebundenen Zellen aus dem Blut herauszuziehen (z. B. an die Wand des Trenngefässes). Das auf diese Weise Ziel-Zellen-depletierte Blut kann gegen andere Lösungen ausgetauscht werden, wobei die über Magnetpartikel separierten Zellen bis zum Abschalten/Entfernen des Magnetfeldes vor Ort verbleiben und für weitere Anwendungen zur Verfügung stehen.

[0026] Alternativ zu den dargestellten Trennprinzipien sind auch jegliche andere Trennprinzipien aus dem Stand der Technik, die auf der Markierung von Zellen mit Antikörpern beruhen, einsetzbar.

[0027] Erfindungsgemäß werden vorteilhafterweise für die Erkennung der Tumorzellen spezifische AntikörperMischungen verwendet. Beispielsweise eignet sich zur Erkennung von Tumorzellen im Blut eine Kombination der Antikörper MOC-31 und Ber-EP4.

Tabelle 1:

| Antikörper-Mischung | | |
|----------------------|-------------------------|---------------------------------|
| Antigen | Klon | Konzentration |
| Epith.Rel.Antigen | MOC-31 (Fa. Novocastra) | 1,25 µl/10 ⁸ Zellen |
| Epitheliales Antigen | Ber-EP 4 (Fa. DAKO) | 0,924 µg/10 ⁶ Zellen |

[0028] Mittels der Antikörpermischung in der vorhergehenden Tabelle 1 werden bevorzugt Tumorzellen, jedoch mit hoher Spezifität erfasst. Dies beruht auf der bevorzugten Expression bestimmter Oberflächenproteine, die Krebszellen von anderen Zellen unterscheidet.

[0029] Derartige Antikörpermischungen zeigen im Vergleich zu den jeweils separat eingesetzten Antikörpern bei der Zellerkennung und Zelltrennung unabhängig von der angewandten Methode eine erhöhte Sensitivität.

[0030] Die vorliegende Erfindung beruht weiterhin wesentlich darauf, dass nicht etwa auf immunologischer oder enzymatischer Ebene Zellmarker im Blut von Patienten nachgewiesen werden, sondern dass als molekularbiologischer Marker die mRNS (Messenger-Ribonukleinsäure) von gesuchten Zellen in einer Probe beispielsweise einer Blutprobe, nachgewiesen wird.

[0031] Da einzelne Marker in therapieabhängiger Weise unterschiedlich exprimiert werden, wird eine Kombination von Tumormarkern untersucht, um alle im Blut zirkulierenden Tumorzellen zu erfassen. Hierdurch lassen sich Tumorzellen auch dann erkennen, wenn die Expression eines bestimmten Markers bei einem Patienten bzw. in einem Krankheitsstadium relativ gering ist, was sonst zu einem vermeintlich negativen Ergebnis führen könnte. Die Verwendung von Markern stößt jedoch meist deswegen auf Grenzen, weil mononukleäre Blutzellen eine Hintergrundexpression ("illegitime Transkription") aufweisen, die eine exakte Analyse behindern.

[0032] Als Marker, beispielsweise für Tumoren, wird die Expression der in Tabelle 2 genannten Gene erfasst. Der Nachweis kann dabei für zwei Marker oder auch für eine beliebige Anzahl dieser Tumormarker in Kombination miteinander durchgeführt werden. Das erfindungsgemäße Kit kann daher Oligonukleotidpaare für zwei oder eine beliebige Auswahl oder auch alle der Tumormarker enthalten.

20

Tabelle 2

| Gen bzw. Genprodukt | Gen | Alternativ-bezeichn.. |
|--|------------------------|-----------------------|
| Human carcinoma-associated antigen GA733-2 gene | GA733-2 | GA733.2 |
| Human epidermal growth factor receptor (EGFR) gene | EGFR | EGFR |
| Human carcinoembryonic antigen (CEA) gene | CEA | CEA |
| Homo sapiens mucin 1 (MUC1) | MUC1 | CA15-3 |
| Homo sapiens C-erb B2/neu protein (ERBB2) gene | HER-2/neu | HER-2 |
| Homo sapiens claudin 7 (CLDN7), mRNA | claudin7 (CLDN7) | Claudin-7 |
| Alkaline phosphatase, placental-like (Nago isozyme), (Germ-cell alkaline phosphatase), (PLAP-like) | ALPPL2 (GCAP) | PLAP |
| Homo sapiens gastrin-releasing peptide receptor (GPPR) gene | GRPR | GRPR |
| Homo sapiens high-mobility group (nonhistone chromosomal) protein isoform I-C (HMGIC), mRNA | HMGIC | HMGIC |
| Homo sapiens gene for cytokeratin 20 | CK20 | CK20 |
| Human MAGE-3 antigen (MAGE-3) gene | MAGE-3 | MAGE-3 |
| Homo sapiens stanniocalcin 1 (STC1) gene | Stanniocalcin 1 (STC1) | Stanniocalcin |

[0033] Dadurch werden alle diejenigen Fälle richtigerweise vernachlässigt, bei denen beispielsweise aufgrund von anderen Krankheiten ebenfalls die Tumormarker exprimiert werden und lediglich als Protein in die Blutbahn gelangen. Es werden folglich aufgrund des ersten immunologischen Auswahlschrittes lediglich Zellen erfasst, die zum einen sich selbst in der Blutprobe befinden und zum anderen den oder die jeweiligen Tumormarker exprimieren. Dabei handelt es sich folglich um Tumorzellen, die aus ihrem ursprünglichen Tumorgewebe stammen und in das Blut der Patienten verschleppt wurden. Da im Blut nicht an einem Tumor Erkrankter die mRNA der untersuchten Marker normalerweise nicht exprimiert wird, zeigt sich eine direkte Korrelation zwischen dem Auftreten der zugehörigen mRNA und einer Metastasierung schon im frühen Stadium im Metastasierungsprozess.

[0034] Dabei wird nicht nur die mRNS eines einzelnen Tumormarkers erfasst sondern eine Kombination von Markern untersucht. Dadurch ist es möglich, Krebsformen über ihre im Blut metastasierenden Zellen erfassen zu können. Dies bedeutet, dass im Falle von Hodentumoren sowohl seminäre als auch nichtseminäre Hodenkrebsformen bzw. auch Mischtumore mit Anteilen eines Seminoms und damit 90-95 % aller malignen Tumore des Hodens, nämlich sämtliche Keimzelltumoren, erfasst werden.

[0035] Für die Erkennung von Hodentumorzellen wird daher erfindungsgemäß eine Kombination von mindestens

zwei der folgenden Marker vorgeschlagen:

- GA733.2
- GCAP/PLAP
- HMGI-C
- GRPR,

[0036] Für die Erkennung von Brustkrebszellen wird erfindungsgemäß eine Kombination von mindestens zwei Tumormarkern der folgenden Markergruppen vorgeschlagen:

- 10 a) EGFR, CEA, Stanniocalcin, MAGE 3, CK20, Claudin 7, Her-2/neu, MUC1 und GA 733.2;
- b) CK20, MAGE 3 und MUC1
- c) Her-2/neu und Claudin7 sowie
- d) EGFR, CEA und Stanniocalcin

[0037] Für die Erkennung von Darmkrebszellen wird erfindungsgemäß eine Kombination von mindestens zwei Tumormarkern der folgenden Markergruppen vorgeschlagen:

- 20 a) CK20, EGFR, GA 733.2, CEA und Stanniocalcin
- b) CK20, EGFR, CEA und Stanniocalcin sowie
- c) EGFR, CEA und GA 733.2

[0038] Im folgenden werden einige Beispiele gegeben, aus denen hervorgeht, daß mit dem erfindungsgemäßen Verfahren eine Nachweissensitivität erzielt wird, die über alles bisher im Stand der Technik bekannte weit hinausgeht.

25 Die Figuren 1 bis 10 zeigen Ergebnisse verschiedener Versuchsprotokolle.

[0039] Gemeinsam für sämtliche Beispiele ist das grundsätzliche Vorgehen, das einen ersten Schritt mit immunologischer Anreicherung von Zielzellen und einen zweiten Schritt eines Nachweises von mRNS-Markern in den immunologisch angereicherten Zellen umfaßt. Im folgenden werden diese Schritte in allgemeiner Form beschrieben, soweit sie für sämtliche Beispiele identisch sind.

30 1. Immunologische Anreicherung der Zielzellen aus peripherem Blut

[0040] Zuerst wurde eine periphere Blutprobe entnommen und dieser eine definierte Anzahl von Zielzellen hinzugegeben, beispielsweise 2, 10, 100 Zellen eines bestimmten Tumortyps.

35 [0041] Weiterhin wurden Antikörper an Magnetpartikel gekoppelt. Als Antikörper wurden dabei die im folgenden in Tabelle 3 dargestellten Antikörper verwendet.

Tabelle 3

| Antigen | Klon | Firma |
|------------------------------|-----------|--------------------------|
| Epitheliales Membran Antigen | GP1.4 | Novocastra |
| Epitheliales Antigen | MOC-31 | Novocastra |
| Epitheliales Antigen | Ber-EP4 | DAKO |
| Muc 1 | HMPV.2 | Pharmingen |
| PLAP | 8B6 | Cymbus Biotechnology LTD |
| Epitheliales Membran Antigen | E29 | DAKO |
| Epitheliales Membran Antigen | 131-11741 | HISS |

[0042] Die Magnetpartikel wurden dabei mit einer Partikelkonzentration von 4×10^8 beads/ml (CELLection™ Pan Mouse IgG Kit, Firma Dynal) verwendet. Die Verhältnisse zwischen der Antikörperkonzentration und den daran gekoppelten Antikörpern sind in Tabelle 4 wiedergegeben.

Tabelle 4

| Klon | Antikörperkonzentration | µl Antikörper/25 µl Partikel |
|-----------|--|------------------------------|
| BerEP4 | 0,1 mg/ml | 4 µl |
| HMPV.2 | 0,5 mg/ml | 4 µl |
| MOC31 | k.A. Verdünnung s. Herstellerangaben (Lyophilisat) | 4 µl |
| GP1.4 | k.A. Verdünnung s. Herstellerangaben (Lyophilisat) | 4 µl |
| 8B6 | 0,1 mg/ml | 1 µl |
| 131-11741 | 0,5 mg/ml | 4 µl |
| E29 | 0,1 mg/ml | 4 µl |

[0043] Die so vorbereiteten Magnetpartikel wurden je nach Versuchsansatz und Nachweissystem dem Blut zugegeben. Der entsprechende Zusatz Antikörper-gekoppelter Magnetpartikel pro ml Blut bei einer Ausgangskonzentration von 4×10^8 beads/ml Partikel ist in Tabelle 5 wiedergegeben.

Tabelle 5

| Tumor Antikörper | Brustkrebs- diagnostik | Darmkrebs- diagnostik | Hodenkrebs- diagnostik |
|---------------------|---------------------------|--------------------------|---------------------------|
| BerEP4 | 8,3 µl | 10 µl | 8 µl |
| HMPV.2 | 8,3 µl | | |
| MOC31 | | 10 µl | 8 µl |
| GP1.4 | 8,3 µl | | |
| 8B6 | | | 4 µl |

[0044] Nach 2-stündiger Inkubation im Überkopfschüttler wurden die Magnetpartikel, die gegebenenfalls als Zell-Antikörper-Magnetpartikelkomplexe vorlagen, mittels eines Magnetpartikelkonzentrators (MPC®-S, Firma Dynal) 3 mal mit PBS (Phosphatpuffer-Saline) gewaschen und die anhaftenden Zellen anschließend entsprechend des im folgenden beschriebenen RNS-Isolierungsprotokolls behandelt.

[0045] Als Alternative zur Abtrennung mittels Magnetpartikeln bietet sich eine immunologische Abtrennung mittels Durchflußzytometrie (Fluoreszenz-assoziierte Zellsortierung, FACS) an.

[0046] Hier wird eine erste relative Anreicherung der Tumorzellen durch Depletion der Erythrozyten erzielt. Dazu wird Vollblut (mit EDTA) mit einem hypotonen Erythrozyten-Lyse-Puffer vermischt und 30 Minuten bei Raumtemperatur inkubiert. Die verbliebenen kernhaltigen Zellen werden zentrifugiert und in PBS/BSA resuspendiert. Die so gewonnenen Zellen werden anschließend mit Antikörpern inkubiert, die mit einem Fluorophor markiert sind. Die Zielzellen, die durch Bindung an einen Antikörper fluoreszierend markiert sind, wurden dann über FACS abgetrennt.

[0047] Alternativ bietet sich eine Anreicherung durch Dichtegradienten-Zentrifugation an. Durch eine derartige Zentrifugation mit unterschiedlichen Dichtegradienten werden Zellen unterschiedlicher mittlerer Volumendichte voneinander getrennt. Mononukleare Blutzellen werden mittels eines Ficoll-Hypaque-Gradienten (Firma Pharmacia, Uppsala, Schweden) separiert und anschließend zweifach mit PBS/1% FCS gewaschen. Anschließend erfolgt eine Festphasen-gekoppelte Anreicherung (z.B. über Magnetpartikel) bzw. eine Flüssigphasen-basierte Abtrennung (FACS) der Zielzellen wie oben beschrieben.

55 2. mRNS-Isolierung

[0048] Als erstes erfolgt eine Isolierung der Gesamt-RNS der wie oben beschrieben abgetrennten Zellen. Diese

erfolgt mit dem QIAamp RNA Blood Mini Kit (Firma Qiagen, Hilden) nach dortigen Herstellerangaben, wobei der Lysis-Puffer direkt zu den an die Magnetpartikel gebundenen Zellen gegeben wurde. Durch einen zusätzlichen DNS-Verdau auf der Säule wird eine Kontamination mit genomicscher DNS vermieden. Dieser DNS-Verdau erfolgt mit dem RNase-Free DNase Set, Firma Qiagen, Hilden.

5 [0049] Alternativ kann auch eine mRNS-Isolierung, z. B. mittels Oligo(dT)-gekoppelter Magnetpartikel, Dynabeads® mRNA Direct™ Micro Kit, (Firma Dynal)) erfolgen. Auch diese Isolation erfolgt entsprechend der in dem Kit angegebenen Herstellerangaben.

10 [0050] Als weitere Alternative zur RNS-Isolierung werden die isolierten Zellen durch Zugabe von Trizol-Reagenz (Firma Gibco BRL, NY, USA) lysiert und mittels Pipette homogenisiert. Nach anschließender Chloroformextraktion wird die RNS-haltige wässrige Phase in Isopropanol bei -80 °C gefällt. Nach zweimaligem Waschen und Zentrifugieren in 80 %igem Ethanol wird das Pellet an der Luft getrocknet und anschließend in RNase freiem Wasser resuspendiert. Dieser Aufarbeitungsschritt erfolgt ebenfalls nach herkömmlichen Protokollen.

3. Reverse Transkription

15 [0051] An die Isolierung der RNS schließt sich eine reverse Transkription an, bei der die mRNS in cDNS umgeschrieben wird.

20 [0052] Dazu wird die RNS in einem entsprechenden Volumen Wasser gemäß dem Reaktionsansatz in Tabelle 6 zusammen mit Oligo(dT)15-Primern (Firma Promega, Mannheim) für 5 Minuten bei 65 °C denaturiert und anschließend direkt auf Eis inkubiert.

Tabelle 6:

| Komponenten der cDNS-Synthese | | |
|--|----------|------------------|
| Die cDNS-Synthese erfolgt in einem 20 µl-Reaktionsansatz | | |
| Komponenten | Volumen | Endkonzentration |
| RNS/mRNS | 10 µl | - |
| 10 x RT-Puffer | 2 µl | 1 x |
| dNTP-Mix (je 5 mM) | 2 µl | jeweils 0.5 mM |
| Oligo(dT)-Primer (10 µM) | 2 µl | 1 µM |
| RNase-Inhibitor | 1 µl | 0.5 Units/µl |
| Reverse Transkriptase | 1 µl | 4 U |
| RNase-freies Wasser | ad 20 µl | |

25 [0053] Die cDNS-Synthese erfolgte bei 37 °C für eine Stunde mit nachfolgender Inaktivierung der reversen Transkriptase durch Erhitzen für 5 Minuten bei 95 °C und anschließender Abkühlung auf Eis. Hierzu wurde ein Sensiskript Reverse Transkriptase Kit, Firma Qiagen, Hilden nach dort angegebenen Protokollen verwendet.

30 [0054] Im Falle der Verwendung Oligo(dT)-gekoppelter Magnetpartikel bereits zur Isolierung von mRNS unterbleibt anschließend die Zugabe von Oligo(dT)-Primern, d.h. der Oligo(dT)-Linker dient zugleich als Primer für die reverse Transkription, wobei hier die Magnetpartikel im Ansatz verbleiben.

45 4. PCR

[0055] Anschließend an die Umschreibung der mRNS in cDNS erfolgt eine Polymerase-Kettenreaktion (PCR) mit β-Aktin als interner Kontrolle.

50 [0056] Dabei wurden die in Tabelle 7 aufgeführten Oligonukleotide als PCR-Primer zur Amplifikation von cDNS entsprechend verschiedener Markergene, wie sie in der ersten Spalte angegeben sind, verwendet.

Tabelle 7: Liste der PCR-Primer

| Primename | Sequenz | 5' → 3' | PCR-Produkt |
|--------------------------|---------------------------|---------|-------------|
| Tumormarker | | | |
| GA733.2 sense | AATCGTCAATGCCAGTGTACTTCA | 395 bp | |
| GA733.2 sense | TAACCGCGTTGTGATCTCCTCTGA | | |
| EGFR sense | AGTCGGGCTCTGGAGGAAAAGAAA | 163 bp | |
| EGFR antisense | GATCATAATTCCCTCTGCACATAGG | | |
| CEA sense | AGAAATGACGCAAGAGCCTATGTA | 231 bp | |
| CEA antisense | AACTTGTGTGTGTTGCTGCGGTAT | | |
| CA15-3 sense | TCAGCTTCTACTCTGGTGACAAC | 299 bp | |
| CA-15-3 antisense | TGGTAGTAGTCGGTGCTGGATCT | | |
| Her-2 sense | CCCAGTGTGTCAACTGCAGCCAGT | 265 bp | |
| Her-2 antisense | CAGATGGGCATGTAGGAGAGGTCA | | |
| Claudin-7 sense | GTCTTGCCTGCCTTGGTAGCTTGCT | 225 bp | |
| Claudin-7 antisense | TGGACTTAGGGTAAGAGCGGGGTG | | |
| GCAP/PLAP sense | GCCACGCAGCTCATCTCCAACATG | 440 bp | |
| GCAP/PLAP antisense | ATGATCGTCTCAGTCAGTGCCCCGG | | |
| GRPR sense | TCTCCCCGTGAACGATGACTGGTC | 308 bp | |
| GRPR antisense | TGAAGACAGACACCCCAACAGAGG | | |
| HMGI-C sense | AAAGGCAGCAAAACAAGAGTCCC | 213 bp | |
| HMGI-C antisense | CCAACTGCTGCTGAGGTAGAAATC | | |
| CK20 sense | ATCTCCAAGGCCTGAATAAGGTCT | 336 | |
| CK20 antisense | CCTCAGTTCTTTAATTCTTCAGT | | |
| MAGE3 sense | CTCCAGCCTCCCCACTACCATGAA | 375 bp | |
| MAGE3 antisense | TTGTCACCCAGCAGGCCATCGTAG | | |
| Stanniocalcin sense | AACCCATGAGGCAGGAGCAGAATGA | 254 bp | |
| Stanniocalcin antisense | CGTTGGCGATGCATTTAAGCTCT | | |
| Interne Kontrolle | | | |
| Aktin sense | CTGGAGAAGAGCTACGAGCTGCCT | 111 bp | |
| Aktin antisense | ACAGGACTCCATGCCAGGAAGGA | | |

[0057] Tabelle 7 enthält in der ersten Spalte die Angabe des zu erfassenden Tumormarkers, wobei jeweils zwei Oligonukleotide (sense und antisense) als Primerpaar angegeben sind. Die Länge des PCR-Produktes, das durch die in Spalte zwei angegebenen Primer erzeugt wird, ist in Spalte drei angegeben. Die PCR wurde mit dem in Tabelle 8 angegebenen Ansatz durchgeführt.

Tabelle 8:

| PCR-Ansatz Die PCR-Synthese erfolgte in einem 50 µl-Rekationsansatz | | | |
|--|----------------------|---------------------|------------------|
| | Komponenten | Volumen | Endkonzentration |
| 5 | cDNS | 6 µl | |
| 10 | 10 x PCR-Puffer* | 5 µl | 1 x |
| | dNTP-Mix | 1 µl | jeweils 200 µM |
| 15 | Primer | s. Tabellen 7 und 9 | |
| | Taq-DNA Polymerase** | 0,5 µl | 2.5 U |
| | [Q-Solution***] | 10 µl] | |
| | H ₂ O | ad 50 µl | |

(* enthält 15 mM MgCl₂; ** HotStarTaq™ DANN Polymerase; Qiagen, Hilden)

(***) Der Zusatz von 10 µl Q-Solution (Qiagen, Hilden) ist nur zum Nachweis von GCAP1PLAP notwendig)

20 [0058] Tabelle 9 gibt eine Auflistung der spezifischen Primerkombination und Primerkonzentrationen als Endkonzentration im PCR-Ansatz an. In den folgenden Beispielen wird für die verschiedenen Tumorarten Brustkrebs, Darmkrebs und Hodenkrebs jeweils exemplarisch eine Multiplexkombination zu diesen Primern aufgezeigt, so wie sie in Tabelle 9 angegeben ist.

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Tabelle 9: Auflistung der spezifischen Primerkombinationen und Primerkonzentration (Endkonzentration im PCR-Ansatz)

| Marker Primer | Brust- krebs-1 | Darm- krebs-1 | Hoden- krebs-1 |
|---------------------|-------------------|------------------|-------------------|
| GA733.2 sense | 500 nM | 500 nM | 500 nM |
| GA733.2 antisense | 500 nM | 500 nM | 500 nM |
| EGFR sense | | 750 nM | |
| EGFR antisense | | 750 nM | |
| CEA sense | | 750 nM | |
| CEA antisense | | 750 nM | |
| CA15-3 sense | 400 nM | | |
| CA15-3 antisense | 400 nM | | |
| Her-2 sense | 300 nM | | |
| Her-2 antisense | 300 nM | | |
| Claudin-7 sense | 400 nM | | |
| Claudin-7 antisense | 400 nM | | |
| GCAP/PLAP sense | | | 800 nM |
| GCAP/PLAP antisense | | | 800 nM |
| GRPR sense | | | 500 nM |
| GRPR antisense | | | 500 nM |
| HMGI-C sense | | | 500 nM |
| HMGI-C antisense | | | 500 nM |
| β-Aktin sense | 100 nM | 200 nM | 100 nM |
| β-Aktin antisense | 100 nM | 200 nM | 100 nM |

[0059] Die PCR-Bedingungen (Zyklenzahl, Zyklenführung etc.) sind in den Tabellen 10 und 11 gegeben.

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Tabelle 10:

| PCR-Bedingungen | | | |
|--------------------|---------------|----------------------------|--|
| Vorabdenaturierung | | 95 °C 15 min | |
| Zyklus | | | |
| 1. | Denaturierung | 94 °C 1 min | |
| 2. | Annealing | x °C 1 min (s. Tabelle 11) | |
| 3. | Extension | 72 °C 1 min | |
| Finale Extension | | 72 °C 10 min | |
| | | 4 °C Pause | |

Tabelle 11:

| Multiplex-spezifische Annealingtemperatur und Zyklenzahl | | | |
|--|--------------|-------------|--------------|
| Marker | Brustkrebs-1 | Darmkrebs-1 | Hodenkrebs-1 |
| Annealing-Temperatur | 60 °C | 58 °C | 58 °C |
| Zyklenzahl | 35 | 40 | 40 |
| (Thermocycler: PCT 200; Biozym) | | | |

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[0060] Die so erzeugten Amplifikate der cDNS wurden elektrophoretisch aufgetrennt mittels eines Bioanalyzer 2100 (Firma Agilent). Hierzu wurde 1 µl des PCR-Produktes in dem Bioanalyzer auf einem DNS-Chip (500) aufgetrennt und das Trennergebnis elektronisch dokumentiert. Auf diese Weise wurden die Figuren 1-10 erzeugt.

[0061] Alternativ werden 25 µl des PCR-Produktes über ein 2,5 %iges Agarosegel aufgetrennt und die DNS-Banden mit Ethidiumbromid angefärbt. Die Dokumentation erfolgt z.B. mit Hilfe des DUO Store Systems der Firma Intas.

[0062] Alternativ kann weiterhin eine Fragmentanalyse beispielsweise mittels eines ABI Prism 310 Genetic Analyser (Firma PE Applied Biosystem, Weiterstadt) durchgeführt werden. Hierzu wird dann jeweils 1 µl des PCR-Produktes in der Verdünnung 1:50 eingesetzt. In diesem Falle werden fluoreszenzmarkierte Primer verwendet.

[0063] Figur 1 zeigt nun das Ergebnis eines erfundungsgemäßen Verfahrens für die Erkennung von Brustkrebszellen im Blut. Hierzu wurde Blut von Gesunden eine definierte Menge an Tumorzellen einer Brustkrebszelllinie zugegeben. Die zugegebene Zellzahl betrug dabei 10 Zellen (10 Z)bzw. 100 Zellen (100 Z)pro Milliliter Blut. Figur 1A und 1B zeigen nun jeweils elektrophoretische Auftrennungen, wobei die einzelnen Banden hier und im folgenden mit denselben Begriffen beschriftet sind. Der Begriff Leiter bezeichnet Calibratoren von 50-600bp Länge, mit RT-Ko ist eine Kontrolle bezeichnet, die keinerlei mRNA enthielt, mit PCR-Ko ist eine Kontrollmessung bezeichnet, die keinerlei cDNS vor der PCR enthielt. Mit "Blut" ist die Blutprobe ohne inkulizierte Tumorzellen, mit 10 Z die Blutprobe mit 10 inkulierten Tumorzellen pro Milliliter und mit 100 Z die Blutprobe mit 100 inkulierten Tumorzellen pro Milliliter bzw. pro 5 Milliliter bezeichnet. Mit "Zelllinie" ist eine Kontrollmessung mit einer großen Zellzahl der Tumorzelllinie in der Probe bezeichnet.

[0064] In Figur 1 sind Ergebnisse dargestellt, wenn die Selektion im ersten Schritt mit lediglich einem, zweien oder allen drei der folgenden Antikörper HMPV.2, GP1.4 und Ber-Ep 4 durchgeführt wurde. Es ist unmittelbar zu erkennen, daß bei Verwendung lediglich eines Antikörpers der Nachweis des Tumormarkers CA 15.3 (Muc1) nur gering ist. Die besten Ergebnisse werden erzielt, wenn zwei der Antikörper, nämlich HMPV.2 und Ber-Ep 4 bzw. GP1.4 und Ber-Ep 4 zum Nachweis eingesetzt werden. Bereits die Kombination aller drei Antikörper ist, wie man an der Intensität der Banden für den Tumormarker CA 15.3 erkennen kann, effektiver. Damit ist nachgewiesen, daß bei geeigneter Auswahl einer bestimmten Anzahl von spezifischen Antikörpern ein erheblich verbessertes Ergebnis beim Nachweis von Tumorzellen möglich ist. Insbesondere zeigt sich auch, daß der simple Schluß, daß unter Einsatz von mehreren Antikörpern die Sensitivität zwangsweise steigen würde, nicht möglich ist. Das Gegenteil ist unter Umständen der Fall, da eine unspezifische Reaktion mit steigender Zahl von Antikörpern eher möglich wird. Es ist daher von besonderer Bedeutung, experimentell eine geeignete Kombination von Antikörpern zu ermitteln.

[0065] Figur 2 zeigt nun Ergebnisse von Nachweisverfahren, bei denen in Figur 2A keine Vorauswahl mittels Antikörpermarkierung und in Figur 2B eine Vorauswahl mittels Antikörpermarkierung durchgeführt wurde. In Figur 2 sind dabei Kombinationen der Antikörper HMPV.2, Ber-Ep 4 und GP1.4 als Zweierkombination bzw. sämtlicher drei Antikörper bestimmt worden. Zugleich wurde eine Multiplexbestimmung von insgesamt vier Markern, nämlich GA 733.2, CA 15.3, Her 2/neu sowie Claudin 7, durchgeführt. Auch hier wurden wieder 10, 100 bzw. 1000 Tumorzellen einer Brustkrebszelllinie in Blut inkuliert und anschließend nachgewiesen. Hier zeigt sich, daß ohne Antikörper-Selektion ein Hintergrundexpression für einige der mRNS-Marker (GA 733.2, CA 15.3 und Her 2/neu) erfaßt wird. Ein derartiger Hintergrund läßt sich bei Einsatz jeder der in Figur 2B dargestellten Antikörperkombinationen zur Vorauswahl der auf mRNS zu untersuchenden Zellen vermeiden. Interessant ist in Figur 2B wiederum, daß der Einsatz von drei Antikörpern dem Einsatz von zwei Antikörpern, z. B. GP 1.4 mit Ber-Ep 4 nicht unbedingt überlegen ist. Die Auswahl bestimmter Antikörperkombinationen sowie die Auswahl bestimmter mRNS-Marker ermöglicht es jedoch, bis herab zu 10 Zellen pro Milliliter Blut ohne jeglichen unspezifischen Hintergrund die entsprechenden gesuchten Tumorzellen zu erfassen. Die Hintergrundexpression konnte eliminiert und die Sensitivität beträchtlich gesteigert werden (s. Bande für Claudin 7).

[0066] In Figur 3 ist das Ergebnis eines erfundungsgemäßen Verfahrens mit in Blut inkulierten Tumorzellen aus einer Hodenkrebszelllinie dargestellt. Wiederum werden sämtliche Tumorzellen aus der Blutprobe selektiert, die durch einen der dargestellten Antikörper markiert sind. Anschließend wird auf insgesamt vier mRNS-Marker (GCAP, GA 733.2, GRPR und HGMI-C) untersucht. Hier zeigt sich, daß bei Einsatz nur eines Antikörpers wie Ber-Ep 4 mittels des Markers HGMI-C lediglich bis herab zu 10 Zellen pro Milliliter Blut erfaßt werden und bei Einsatz des Antikörpers MOC-31 überhaupt keine Hodenkrebszellen erfaßt werden. Dasselbe gilt für den Antikörper 8B6, der lediglich eine geringe

Sensitivität aufweist.

[0067] Die Kombination der Antikörper Ber-Ep 4 und 8B6 führt ebenfalls zu einem mangelhaften Nachweis mittels des Markers HGMI-C sowie auch die Kombination der Antikörper Ber-Ep 4 und MOC-31. Ein optimales Nachweisergebnis für sämtliche vier untersuchten Marker ergibt sich für Hodentumorzellen bei Einsatz von insgesamt drei Antikörpern Ber-Ep 4, MOC-31 und 8B6, wo bis herab zu 2 Zellen pro Milliliter Blut sicher über jeden einzelnen der Marker nachgewiesen werden. Werden erfindungsgemäß mit den zwei Nachweisreaktionen zwei Marker nachgewiesen, so läßt sich bei Auswahl von zwei Markern aus den für Figur 3 verwendeten Markern ein sicherer Nachweis einer minimalen Zellenzahl bei gleichzeitiger Vermeidung einer Hintergrunderfassung durchführen.

[0068] Figur 4 zeigt den Nachweis von Darmkrebszellen, die in Blut eines Gesunden inkuliert wurden. Hier zeigt sich unmittelbar, daß der Einsatz einer Kombination der Antikörper Ber-Ep 4 und MOC-31 zu einer verbesserten Empfindlichkeit bezüglich des mRNS-Markers EGF-R führt. Beim Einsatz der beiden Antikörper gleichzeitig wird eine Nachweisempfindlichkeit von 100 Zellen pro Milliliter Blut erreicht, während bei Einsatz lediglich eines Antikörpers die Empfindlichkeit bei ca. 1000 Zellen pro Milliliter Blut liegt.

[0069] Figur 5 zeigt einen Versuch, bei dem mittels einer Antikörperkombination von Ber-Ep 4, HMPV.2 und GP1.4, die mit zumindest einem der Antikörper markierten Zellen selektiert wurden und anschließend auf die mRNS-Marker GA 733.2, CA 15.3, Her 2 und Claudin 7 untersucht wurden. Allerdings wurde hier dem Blut der Gesunden keine Tumorzelle zugegeben sondern definierte Mengen von Epithelialzellen. Wie aus Figur 5 unmittelbar hervorgeht, zeigen lediglich zwei der Marker bei einer sehr hohen Zahl von epithelialen Zellen ein positives Ergebnis.

[0070] In Figur 6 wurden Tumorzellen zweier verschiedener Brustkrebszelllinien (MCF-7 und SKBR-3) einer Blutprobe zugegeben. Als Antikörper wurden die Antikörper Ber-Ep 4, HMPV.2 und GP1.4 in Kombination eingesetzt. Wie unmittelbar zu erkennen ist, wird durch den Einsatz der vier mRNS-Marker GA 733.2, CA 15.3, Her 2 und Claudin 7 jeweils gesichert bis herab zu 10 Zellen der einzelnen Zelllinien pro Milliliter eine Erkennung der Brustkrebszellen gewährleistet. Eine unspezifische Reaktion in Blut ohne Brustkrebszellen trat nicht auf. Allerdings sind die Tumormarker GA 733.2 und CA 15.3 für die Zelllinie 2 (SKBR-3) sensitiver, während der Tumormarker Her 2 für die Zelllinie 1 (MCF-7) sensitiver ist. So können dann auch die einzelnen Untertypen von Brustkrebszellen anhand des aufgetretenen Markermusters voneinander unterschieden werden.

[0071] Auch in Figur 7 sind Brustkrebszellen verschiedener Zelllinien (MCF-7) in Figur 7A und SKBR 3 in Figur 7B in Blut inkuliert worden. Als Antikörper zur Selektion der Zellen aus der Blutprobe wurden die Antikörper Ber-Ep 4, HMPV.2 und GPI.4 in Kombination verwendet. Es ist unmittelbar zu erkennen, daß bei Einsatz einer Kombination der mRNA-Marker GA 733.2, CA 15.3 Her 2 und Claudin 7 in jedem Falle mindestens einer der Marker bis herab zu 2 Zellen pro 5 Milliliter positiv reagiert, ohne daß das Blut ohne Tumorzellen einen Expressionshintergrund liefern würde. Auch hier ist wieder ein differenzielles Ansprechverhalten der beiden Zelllinien auf die vier unterschiedlichen mRNS-Marker zu erkennen.

[0072] Befinden sich beispielsweise jedoch in einer Blutprobe beide Zelllinien, so wäre durch die gewählt Marker-kombination in jedem Falle bis herab von 2 Zellen pro 5 Milliliter Blut eine Erkennung beider Zelllinien gewährleistet, d. h. der Nachweis von Brustkrebszellen im Blut wäre unabhängig vom Zelltyp der Brustkrebszelllinie mit hoher Sensitivität möglich.

[0073] Figur 8 zeigt den Nachweis von Darmkrebszellen, die in Blut inkuliert wurden. Dabei erfolgte die Selektion der Zellen mit den zwei Antikörpern Ber-Ep 4 und MOC-31. Der molekularbiologische Nachweisschritt erfolgte mit den mRNS-Markern GA 733.2, CEA und EGF-R. Zwei Tumorzellen waren in 5 Milliliter Blut nachweisbar.

[0074] Figur 9 zeigt wiederum eine Messung mit einer Kombination aus drei Antikörpern Ber-Ep 4, MOC-31 und 8B6 sowie den mRNA-Markern GCAP/PLAP, GA 733.2, GRPR und HMGI-C an Blut, in das Hodenkrebszellen inkuliert wurden.

[0075] Mit jedem der molekularbiologischen Marker gelingt bei Einsatz dieser Dreierkombination von Antikörpern für die Zellselektionierung im immunologischen Selektionierungsschritt der Nachweis bis herab zu 2 Zellen pro 5 Milliliter.

[0076] Figur 10 zeigt abschließend die Abtrennung von seltenen Zellen aus dem Zellgemisch eines Biopsiematerials. Hierzu wurden Biopsiematerial aus Brustgewebe, das Tumorgewebe mit einem vermuteten Primärtumor enthielt, mechanisch vereinzelt und über Gaze von Zelltrümmern, Bindegewebe etc. getrennt. Das gewonnene Zellgemisch, das sowohl Zellen des vermuteten Tumorgewebes als auch Zellen des umliegenden gesunden Gewebes enthielt, wurde einer Zellselektion mit einem an eine Festphase gekoppelten Antikörpermisch (Magnetpartikel mit Antikörpern GP1.4, HMPV.2 und Ber-Ep 4) versetzt und nach Inkubation zur Herstellung der Antigen-Antikörperbindung magnetisch-separiert. Anschließend erfolgte ein mRNS-Nachweis bezüglich der Marker GA 733.2 und Her 2. Als Kontrolle wurde zugleich eine Zelllinie eines Brustkrebses parallel ebenfalls bestimmt. Wie zu erkennen ist, erfolgt ein positiver Nachweis, daß die Biopsie tatsächlich einen Brustkrebs-Tumor enthielt.

[0077] Die Banden, die in den Figuren 8 und 9 mit "Positivkontrolle" gekennzeichnet sind, zeigen Ergebnisse von Proben mit der Zelllinien HT 29 für Darztumor (Fig. 8) bzw. Tera/l für Hodentumor (Fig. 9).

Patentansprüche

1. Verfahren zum qualitativen und/oder quantitativen Nachweis von vorbestimmten biologischen Zellen aus bzw. in einer biologische Zellen enthaltenden Probe, wobei
 - 5 die Probe mit einer vorbestimmten Kombination von mindestens zwei Antikörpern und/oder Antikörperderivaten, die mit ihren Bindungsstellen an verschiedene auf den nachzuweisenden Zellen präferentiell vorhandene Epitope binden, und/oder mit mindestens einem bispezifischen Antikörper und/oder Antikörperderivat, der/das mit seinen beiden Bindungsstellen an unterschiedliche auf den nachzuweisenden Zellen präferentiell vorhandene Epitope bindet, versetzt wird,
 - 10 die mit mindestens einem der Antikörper und/oder Antikörperderivate markierten Zellen aus der Probe abgetrennt werden,
 - 15 und die abgetrennten Zellen mit einer vorbestimmten Kombination mindestens zweier molekularbiologischer Nachweisreagenzien auf die Expression einer vorbestimmten Kombination mindestens zweier mRNA-Abschnitte geprüft werden, deren Expression in den nachzuweisenden Zellen präferentiell erfolgt, und erfasst wird, ob zumindest einer der mRNA-Abschnitte exprimiert ist.
2. Verfahren nach dem vorhergehenden Anspruch **dadurch gekennzeichnet, dass** die Abtrennung der markierten Zellen in Flüssigphase oder Festphase erfolgt.
- 20 3. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** auf Festphasen gekoppelte Antikörper oder Antikörperderivate verwendet werden, um die Zielzellen aus der Probe abzutrennen.
- 25 4. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** mit Fluorophoren markierte Antikörper oder Antikörperderivate verwendet werden und die Abtrennung der markierten Zellen aus der Probe mittels Durchflußzytometrie (fluoreszenzassoziierte Zelltrennung, FACS) erfolgt..
- 30 5. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet; dass** mit magnetischen bzw. pseudomagnetischen Partikeln gekoppelte Antikörper oder Antikörperderivate verwendet werden und zur Abtrennung der markierten Zellen aus der Probe die magnetischen bzw. pseudomagnetischen, antikörpergekoppelten Partikel nach der Mischung mit der Probe magnetisch von der Probe getrennt werden.
- 35 6. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die Antikörper oder Antikörperderivate Bindungsstellen aufweisen, die an Tumorzellen binden.
- 40 7. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die Antikörper oder Antikörperderivate Bindungsstellen aufweisen, die an Zellen eines oder mehrerer bestimmter Tumortypen oder -untertypen binden.
- 45 8. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** zur Abtrennung von Tumorzellen oder Zellen eines bestimmten Tumortyps oder -subtyps die Antikörper oder Antikörperderivate Bindungsstellen aufweisen, die an Epitope eines epithelialen Antigens, eines epithelialen Membranantigens, des Antigens MUC1 und/oder des Antigens PLAP binden.
- 50 9. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** mindestens einer der Antikörper GP1.4, MOC-31, Ber-EP4, HMPV.2, 8B6, E29 und/oder 131-11741 verwendet wird.
10. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** zur Abtrennung von Tumorzellen allgemein oder eines bestimmten Typs oder Subtyps eine Kombination von Antikörpern verwendet wird, die die Antikörper Ber-EP4 und MOC31 oder mindestens zwei der Antikörper HMPV.2, GP1.4 und Ber-EP4 enthält.
- 55 11. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** zur Abtrennung von Brusttumorzellen eine Kombination von Antikörpern verwendet wird, die mindestens zwei der Antikörper 131-11741, GP1.4, E29 und HMPV.2 oder mindestens zwei der Antikörper HIKEIV.2, GP1.4 und Ber-EP4 enthält.
12. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** zur Abtrennung von Darmtumorzellen eine Kombination von Antikörpern verwendet wird, die die Antikörper Ber-EP4 und MOC-31 enthält.

13. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** zur Abtrennung von Hodentumorzellen eine Kombination von Antikörpern verwendet wird, die mindestens zwei der Antikörper MOC31, Ber-EP4 und 8B6 enthält.
- 5 14. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** zum Nachweis von Tumorzellen oder Zellen eines bestimmten Tumortyps oder -subtyps eine Kombination von mRNS-Abschnitten geprüft wird, die mRNS-Abschnitte korrespondierend zu Sequenzabschnitten mindestens zwei der Gene GA733.2, EGFR, CEA, HER2/neu, Claudin-7 (CLDN7), GCAP(ALPPL2)/ALPP, GRPR, HMGIC, CK20, MAGE3, MUC1 und Stanniocalcin (STC1) enthält.
- 10 15. Verfahren nach dem vorhergehenden Anspruch, **dadurch gekennzeichnet, dass** zum Nachweis von Tumorzellen oder Zellen eines bestimmten Tumortyps oder -subtyps eine Kombination von mRNS-Abschnitten geprüft wird, die mRNS-Abschnitte korrespondierend zu Sequenzabschnitten mindestens zwei der Gene EGFR, GA733.2 und HER-2/NEU enthält.
- 15 16. Verfahren nach Anspruch 14, **dadurch gekennzeichnet, dass** zum Nachweis von Brusttumorzellen eine Kombination von mRNS-Abschnitten verwendet wird, die mRNS-Abschnitte korrespondierend zu Sequenzabschnitten mindestens zwei der Gene GA733.2, MUC1, Her-2/neu, Claudin7, CK20, PIAGE3, Stanniocalcin, EGFR und CEA enthält.
- 20 17. Verfahren Anspruch 16, **dadurch gekennzeichnet, dass** zum Nachweis von Brusttumorzellen eine Kombination von mRNS-Abschnitten verwendet wird, die mRNS-Abschnitte korrespondierend zu Sequenzabschnitten der beiden Gene GA733.12 und MUC1, korrespondierenden zu Sequenzabschnitten der beiden Gene Her-2/neu und Claudin7, korrespondierend zu Sequenzabschnitten mindestens zwei der Gene CK20, MAGE-3 und MUC1 und/oder korrespondierend zu Sequenzabschnitten mindestens zwei der Gene Stanniocalcin, EGFR und CEA enthält.
- 25 18. Verfahren nach Anspruch 14, **dadurch gekennzeichnet, dass** zum Nachweis von Darmtumorzellen eine Kombination von mRNS-Abschnitten verwendet wird, die mRNS-Abschnitte korrespondierend zu Sequenzabschnitten mindestens zwei der Gene CK20, EGFR, GA733.2, CEA und Stanniocalcin enthält.
- 30 19. Verfahren nach Anspruch 18, **dadurch gekennzeichnet, dass** zum Nachweis von Darmtumorzellen eine Kombination von mRNS-Abschnitten verwendet wird, die mRNS-Abschnitte korrespondierend zu Sequenzabschnitten mindestens zwei der Gene CK20, EGFR, CEA und Stanniocalcin und/oder korrespondierend zu Sequenzabschnitten mindestens zwei der Gene EGFR, CEA und GA733.2 enthält.
- 35 20. Verfahren nach Anspruch 14, **dadurch gekennzeichnet, dass** zum Nachweis von Hodentumorzellen eine Kombination von mRNS-Abschnitten verwendet wird, die mRNS-Abschnitte korrespondierend zu Sequenzabschnitten mindestens zwei der Gene ALPP/ALPPL2 (GCAP), CGA733.2(=EGP-40), HMGI-C, GRPR enthält.
- 40 21. Verfahren, nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die mRNS-Abschnitte unter Verwendung von Polymerasekettenreaktion (PCR), LCR, NASBA RT-PCR und/oder Hybridisierungsverfahren vervielfältigt und/oder nachgewiesen werden.
- 45 22. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die mRNS der abgetrennten Zellen in cDNA revers transkribiert, die cDNA vervielfältigt und anschließend das Vorhandensein oder Fehlen des nachzuweisenden mRNS-Abschnittes erfasst wird.
- 50 23. Verfahren nach dem vorhergehenden Anspruch, **dadurch gekennzeichnet, dass** die vervielfältigte cDNA durch geeignete Restriktionsenzyme verdaut und anhand der erzeugten cDNA-Bruchstücke das Vorhandensein oder Fehlen der nachzuweisenden mRNS erfasst wird (Fragmentanalyse).
- 24. Verfahren nach einem der beiden vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die der nachzuweisenden mRNS entsprechende cDNA mittels fluoreszenzbasierter Echtzeit-PCR bestimmt wird.
- 55 25. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** als interne Kontrolle die mRNS des Proteins β-Aktin erfasst wird.
26. Verwendung eines Verfahrens nach einem der vorhergehenden Ansprüche zum Nachweis von Zellen seltenen

Typs in Suspensionen und Zellmischungen, insbesondere von Tumorzellen, Epithelzellen und/oder Endothelzellen in Körperflüssigkeiten, peripherem Blut, Sputum, Ascites, Lymphe, Urin, Knochenmark und/oder Biopsiematerial und/oder von fötalen Zellen in Amnionflüssigkeit oder maternalem peripherem Blut.

- 5 27. Verwendung nach dem vorhergehenden Anspruch zur Diagnostik und/oder Behandlungskontrolle von Tumorerkrankungen.
- 10 28. Verwendung nach dem vorhergehenden Anspruch zur Diagnostik und/oder Behandlungskontrolle von Hodentumor, Brusttumor und/oder Darmtumor.

10

Claims

1. Method for qualitative and/or quantitative detection of predetermined biological cells from or in a sample containing biological cells, wherein
15 the sample is mixed with a predetermined combination of at least two antibodies and/or antibody derivatives, which bind with their binding sites to different epitopes which are preferentially present on the cells to be detected, and/or with at least one bispecific antibody and/or antibody derivative, which binds with its two binding sites to different epitopes which are preferentially present on the cells to be detected,
20 the cells marked with at least one of the antibodies and/or antibody derivatives are separated from the sample, and the separated cells are tested with a predetermined combination of at least two molecular-biological detection reagents for the expression of a predetermined combination of at least two mRNA portions, the expression of which is effected preferentially in the cells to be detected, and
25 it is detected whether at least one of the mRNA portions is expressed.
2. Method according to the preceding claim, **characterised in that** the separation of the marked cells is effected in liquid phase or solid phase.
3. Method according to one of the preceding claims, **characterised in that** antibodies or antibody derivatives coupled to solid phases are used in order to separate the target cells from the sample.
4. Method according to one of the preceding claims, **characterised in that** antibodies or antibody derivatives marked with fluorophores are used and the separation of the marked cells from the sample is effected by means of flow cytometry (fluorescence-associated cell separation, FACS).
5. Method according to one of the preceding claims, **characterised in that** antibodies or antibody derivatives coupled to magnetic or pseudo-magnetic particles are used and in order to separate the marked cells from the sample, the magnetic or pseudo-magnetic antibody-coupled particles after mixing with the sample are separated magnetically from the sample.
6. Method according to one of the preceding claims, **characterised in that** the antibodies or antibody derivatives have binding sites which bind to tumour cells.
7. Method according to one of the preceding claims, **characterised in that** the antibodies or antibody derivatives have binding sites which bind to cells of one or more specific tumour types or sub-types.
8. Method according to one of the preceding claims, **characterised in that**, in order to separate tumour cells or cells of a specific tumour type or sub-type, the antibodies or antibody derivatives have binding sites which bind to epitopes of an epithelial antigen, of an epithelial membrane antigen, of the antigen MUC1 and/or of the antigen PLAP.
9. Method according to one of the preceding claims, **characterised in that** at least one of the antibodies GP1.4, MOC-31, Ber-EP 4, HMPV.2, 8B6, E29 and/or 131-11741 is used.
- 55 10. Method according to one of the preceding claims, **characterised in that**, in order to separate tumour cells in general or of a specific type or sub-type, a combination of antibodies is used, which contains the antibodies Ber-EP4 and MOC31 or at least two of the antibodies HMPV.2, GP1.4 and Ber-EP4.

11. Method according to one of the preceding claims, **characterised in that**, in order to separate breast tumour cells, a combination of antibodies is used, which contains at least two of the antibodies 131-11741, GPI.4, E29 and HMPV.2 or at least two of the antibodies HMEIV.2, GPI.4 and Ber-EP4.
- 5 12. Method according to one of the preceding claims, **characterised in that**, in order to separate colon tumour cells, a combination of antibodies is used, which contains the antibodies Ber-EP4 and MOC-31.
- 10 13. Method according to one of the preceding claims, **characterised in that**, in order to separate testicular tumour cells, a combination of antibodies is used, which contains at least two of the antibodies MOC31, Ber-EP4 and 8B6.
14. Method according to one of the preceding claims, **characterised in that**, in order to detect tumour cells or cells of a specific tumour type or sub-type, a combination of mRNA portions is tested, which contains mRNA portions corresponding to sequence portions of at least two of the genes GA733.2, EGFR, CEA, HER2/neu, claudin-7 (CLDN7), GCAP (ALPPL2)/ALPP, GRPR, HMGIC, CK20, MAGE3, MUC1 and stanniocalcin (STC1).
- 15 15. Method according to the preceding claim, **characterised in that**, in order to detect tumour cells or cells of a specific tumour type or sub-type, a combination of mRNA portions is tested, which contains mRNA portions corresponding to sequence portions of at least two of the genes EGFR, GA733.2 and HER-2/NEU.
- 20 16. Method according to claim 14, **characterised in that**, in order to detect breast tumour cells, a combination of mRNA portions is used, which contains mRNA portions corresponding to sequence portions of at least two of the genes GA733.2, MUC1, Her-2/neu, claudin7, CK20, PIAGE3, stanniocalcin, EGFR and CEA.
- 25 17. Method according to claim 16, **characterised in that**, in order to detect breast tumour cells, a combination of mRNA portions is used, which contains mRNA portions corresponding to sequence portions of both genes GA733.12 and MUC1, corresponding to sequence portions of both genes Her-2/neu and claudin7, corresponding to sequence portions of at least two of the genes CK20, MAGE-3 and MUC1 and/or corresponding to sequence portions of at least two of the genes stanniocalcin, EGFR and CEA.
- 30 18. Method according to claim 14, **characterised in that**, in order to detect colon tumour cells, a combination of mRNA portions is used, which contains mRNA portions corresponding to sequence portions of at least two of the genes CK20, EGFR, GA733.2, CEA and stanniocalcin.
- 35 19. Method according to claim 18, **characterised in that**, in order to detect colon tumour cells, a combination of mRNA portions is used, which contains mRNA portions corresponding to sequence portions of at least two of the genes CK20, EGFR, CEA and stanniocalcin and/or corresponding to sequence portions of at least two of the genes EGFR, CEA and GA733.2.
- 40 20. Method according to claim 14, **characterised in that**, in order to detect testicular tumour cells, a combination of mRNA portions is used, which contains mRNA portions corresponding to sequence portions of at least two of the genes ALPP/ALPPL2 (GCAP), CGA733.2 (=EGP-40), HMGI-C, GRPR.
- 45 21. Method according to one of the preceding claims, **characterised in that** the mRNA portions are multiplied and/or detected using polymerase chain reaction (PCR), LCR, NASBA RT-PCR and/or hybridisation methods.
22. Method according to one of the preceding claims, **characterised in that** the mRNA of the separated cells is transcribed reversely into cDNA, the cDNA is multiplied and subsequently the presence or absence of the mRNA portion to be detected is detected.
- 50 23. Method according to the preceding claim, **characterised in that** the multiplied cDNA is digested by suitable restriction enzymes and the presence or absence of the mRNA to be detected is detected by means of the produced cDNA-fragments (fragment analysis).
24. Method according to one of the two preceding claims, **characterised in that** the cDNA corresponding to the mRNA to be detected is determined by means of fluorescence-based real time-PCR.
- 55 25. Method according to one of the preceding claims, **characterised in that** the mRNA of the protein β-actin is detected as internal control.

26. Use of a method according to one of the preceding claims for detecting cells of an uncommon type in suspensions and cell mixtures, in particular tumour cells, epithelial cells and/or endothelial cells, in body fluids, peripheral blood, sputum, ascites, lymph, urine, bone marrow and/or biopsy material and/or foetal cells in amniotic fluid or maternal peripheral blood.

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27. Use according to the preceding claim for diagnostics and/or treatment control of tumour diseases.

28. Use according to the preceding claim for diagnostics and/or treatment control of testicular tumour, breast tumour and/or colon tumour.

10

Revendications

1. Procédé de détection qualitative et/ou quantitative de cellules biologiques prédéterminées dans un échantillon contenant des cellules biologiques, dans lequel l'échantillon est mélangé à une combinaison prédéterminée d'au moins deux anticorps et/ou dérivés d'anticorps, qui se fixent, par leurs points de fixation, sur divers épitopes présents préférentiellement sur les cellules à détecter, et/ou à au moins un anticorps et/ou un dérivé d'anticorps bispécifique, qui se fixe, par ses deux points de fixation, sur différents épitopes présents préférentiellement sur les cellules à détecter,

20 les cellules marquées par au moins un des anticorps et/ou des dérivés d'anticorps sont séparées de l'échantillon,

et les cellules séparées sont testées avec une combinaison prédéterminée d'au moins deux réactifs de détection de biologie moléculaire pour déceler l'expression d'une combinaison prédéterminée d'au moins deux segments d'ARNm dont l'expression s'effectue de préférence dans les cellules à détecter, et

25 on détecte si au moins un des segments d'ARNm est exprimé.

2. Procédé selon la revendication précédente, **caractérisé en ce que** la séparation des cellules marquées s'effectue en phase liquide ou en phase solide.

30 3. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce qu'on utilise** des anticorps ou des dérivés d'anticorps couplés à des phases solides pour séparer les cellules cibles de l'échantillon.

4. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce qu'on utilise** des anticorps ou des dérivés d'anticorps marqués avec des fluorophores et **en ce que** la séparation des cellules marquées de l'échantillon s'effectue par cytométrie débitmétrique (séparation cellulaire associée à la fluorescence, FACS).

35 5. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce qu'on utilise** des anticorps ou des dérivés d'anticorps couplés avec des particules magnétiques ou pseudomagnétiques, et
en ce que, pour séparer les cellules marquées de l'échantillon, on sépare magnétiquement de l'échantillon les particules magnétiques ou pseudomagnétiques couplées avec les anticorps après mélange avec l'échantillon.

40 6. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que** les anticorps ou les dérivés d'anticorps présentent des points de fixation qui se fixent sur des cellules tumorales.

45 7. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que** les anticorps ou les dérivés d'anticorps présentent des points de fixation qui se fixent sur des cellules d'un ou plusieurs types ou sous-types déterminés de tumeur.

50 8. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que**, pour séparer des cellules tumorales ou des cellules d'un type ou d'un sous-type déterminé de tumeur, les anticorps ou les dérivés d'anticorps présentent des points de fixation qui se fixent sur des épitopes d'un antigène épithéial, d'un antigène membranaire épithéial,-de-l'antigène-MUC1 et/ou-de-l'antigène PLAP.

55 9. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce qu'on utilise** au moins un des anticorps GP1.4, MOC-31, Ber-EP4, HMPV.2, 8B6, E29 et/ou 131-11741.

10. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que**, pour séparer des cellules tumorales de manière générale ou d'un type ou d'un sous-type déterminé, on utilise une combinaison d'anticorps

qui contient les anticorps Ber-EP4 et M0C31 ou au moins deux des anticorps HMPV.2, GP1.4 et Ber-EP4.

- 5 11. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que**, pour séparer des cellules de tumeurs du sein, on utilise une combinaison d'anticorps qui contient au moins deux des anticorps 131-11741, GP1.4, E29 et HMPV.2 ou au moins deux des anticorps HMEIV.2, GP1.4 et Ber-EP4.
- 10 12. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que**, pour séparer des cellules de tumeur intestinale, on utilise une combinaison d'anticorps qui contient les anticorps Ber-EP4 et MOC-31.
- 15 13. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que**, pour séparer des cellules de tumeurs testiculaires, on utilise une combinaison d'anticorps qui contient au moins deux des anticorps MOC31, Ber-EP4 et 8B6.
- 20 14. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que**, pour détecter des cellules tumorales ou des cellules d'un type ou sous-type prédéterminé de tumeur, on teste une combinaison de segments d'ARNm qui contient des segments d'ARNm correspondant à des segments de séquence d'au moins deux des gènes GA733.2, EGFR, CEA, HER2/neu, Claudin-7 (CLDN7), GCAP(ALPPL2)/ALPP, GRPR, HMIGIC, CK20, MAGE3, MUC1 et stanniocalcine (STC1).
- 25 15. Procédé selon la revendication précédente, **caractérisé en ce que**, pour détecter des cellules tumorales ou des cellules d'un type ou d'un sous-type déterminé de tumeur, on teste une combinaison de segments d'ARNm qui contient des segments d'ARNm correspondant à des segments de séquence d'au moins deux des gènes EGFR, GA733.2 et HER-2/NEU.
- 30 16. Procédé selon la revendication 4, **caractérisé en ce que**, pour détecter des cellules tumorales du sein, on emploie une combinaison de segments d'ARNm qui contient des segments d'ARNm correspondant à des segments de séquence d'au moins deux des gènes GA733.2, MUC1, Her-2/neu, Claudiin7, CK20, PIAGE3, stanniocalcine, EGFR et CEA.
- 35 17. Procédé selon la revendication 16, **caractérisé en ce que**, pour détecter des cellules tumorales du sein, on utilise une combinaison de segments d'ARNm qui contient des segments d'ARNm correspondant à des segments de séquence des deux gènes GA733.12 et MUC1, correspondant à des segments de séquence des deux gènes Her-2/neu et Claudiin7, correspondant à des segments de séquence d'au moins deux des gènes CK20, MAGE-3 et MUC1 et/ou correspondant à des segments de séquence d'au moins deux des gènes stanniocalcine, EGFR et CEA.
- 40 18. Procédé selon la revendication 14, **caractérisé en ce que**, pour détecter des cellules de tumeurs intestinales, on emploie une combinaison de segments d'ARNm qui contient des segments d'ARNm correspondant à des segments de séquence d'au moins deux des gènes CK20, EGFR, GA733.2, CEA et stanniocalcine.
- 45 19. Procédé selon la revendication 18, **caractérisé en ce que**, pour détecter des cellules de tumeurs intestinales, on utilise une combinaison de segments d'ARNm qui contient des segments d'ARNm correspondant à des segments de séquence d'au moins deux des gènes CK20, EGFR, CEA et stanniocalcine et/ou correspondant à des segments de séquence d'au moins deux des gènes EGFR, CEA et GA733.2.
- 50 20. Procédé selon la revendication 14, **caractérisé en ce que**, pour détecter des cellules de tumeurs testiculaires, on emploie une combinaison de segments d'ARNm qui contient des segments d'ARNm correspondant à des segments de séquence d'au moins deux des gènes ALPP/ALPPL2 (GCAP), CGA733.2 (=EGP-40), HMGI-C et GRPR.
- 55 21. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que** l'on réplique et/ou détecte les segments d'ARNm en utilisant une amplification en chaîne par polymérase (PCR), LCR, NASBA RT-PCR et/ou un procédé d'hybridation.
- 60 22. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que** les ARNm des cellules séparées sont transcrits à l'inverse dans des ADNc et les ADNc sont répliqués et, ensuite, on décèle la présence ou l'absence du segment d'ARNm à détecter.
- 65 23. Procédé selon la revendication précédente, **caractérisé en ce que** l'ADNc répliqué est digéré par des enzymes

de restriction appropriées et la présence ou l'absence des ARNm à détecter est décelée à l'aide des fragments d'ADNc produits (analyse fragmentaire).

24. Procédé selon l'une quelconque des deux revendications précédentes, **caractérisé en ce que l'ADNc correspondant à l'ARNm à détecter est déterminé au moyen d'une PCR en temps réel à base fluorescente.**
25. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce qu'on décèle comme témoin interne l'ARNm de la protéine β-actine.**
- 10 26. Utilisation d'un procédé selon l'une quelconque des revendications précédentes pour détecter des cellules d'un type rare dans des suspensions et des mélanges cellulaires, en particulier de cellules tumorales, de cellules épithéliales et/ou de cellules endothéliales dans des liquides corporels, le sang périphérique, des expectorations, des ascites, de la lymphe, de l'urine, la moelle osseuse et/ou un matériau biopsique et/ou des cellules foetales dans le liquide amniotique ou le sang périphérique maternel.
- 15 27. Utilisation selon la revendication précédente pour le diagnostic et/ou le contrôle de traitement de maladies tumorales.
- 20 28. Utilisation selon la revendication précédente pour le diagnostic et/ou le contrôle de traitement de tumeurs testiculaires, de tumeurs du sein et/ou de tumeurs intestinales.

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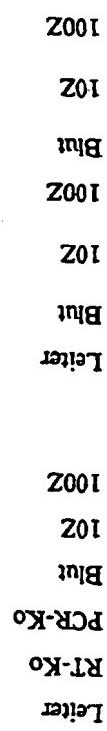
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50

55

A

Einzelne Antikörper

B

Antikörerkombinationen

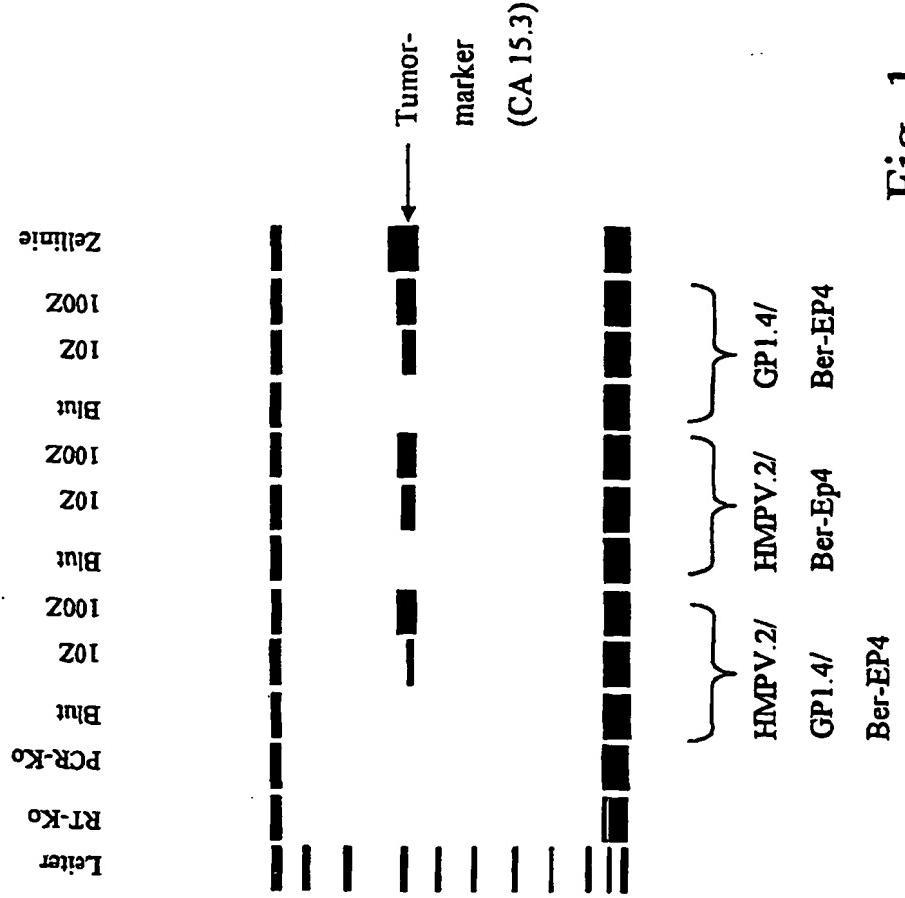


Fig. 1

A |

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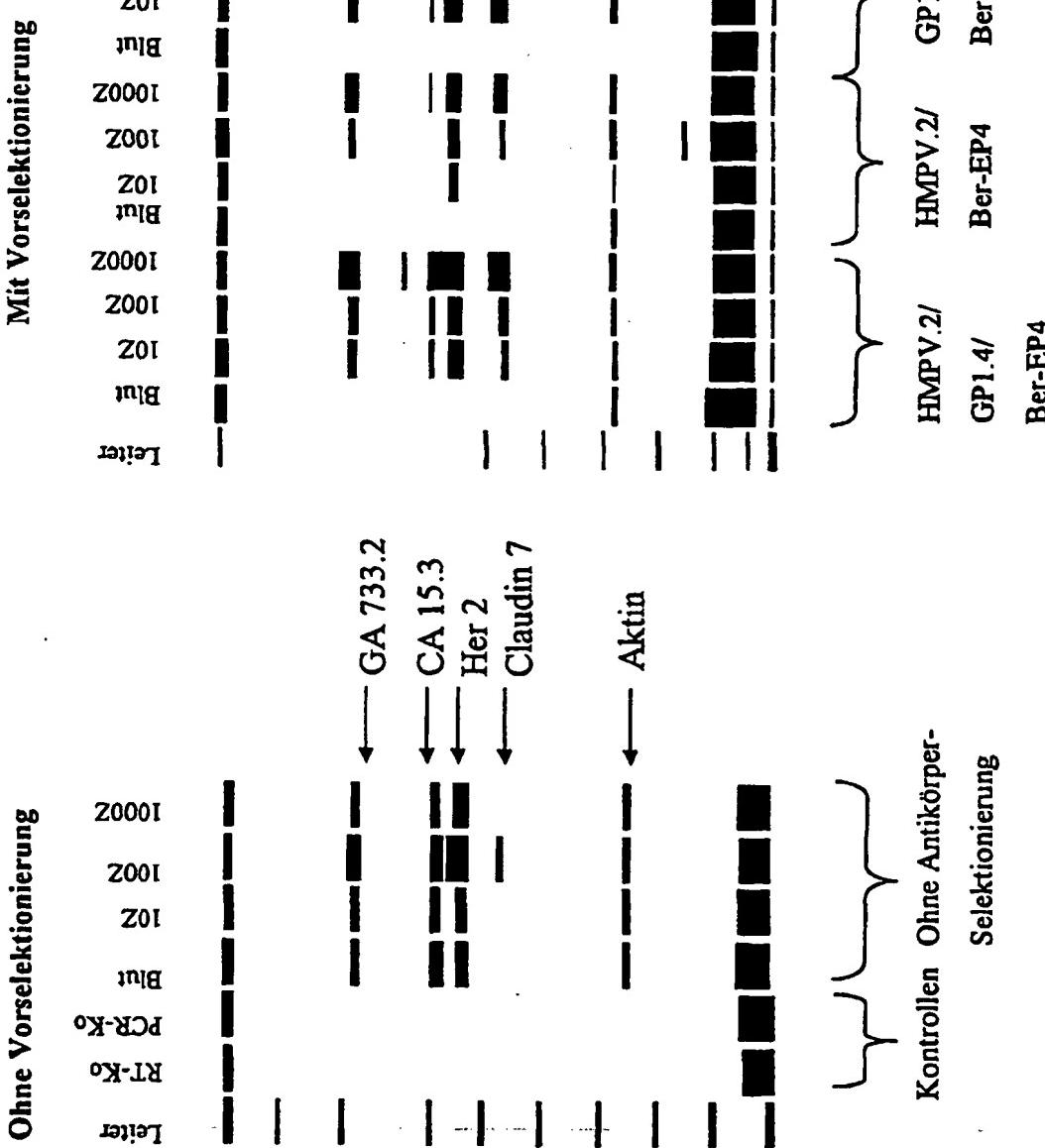


Fig. 2

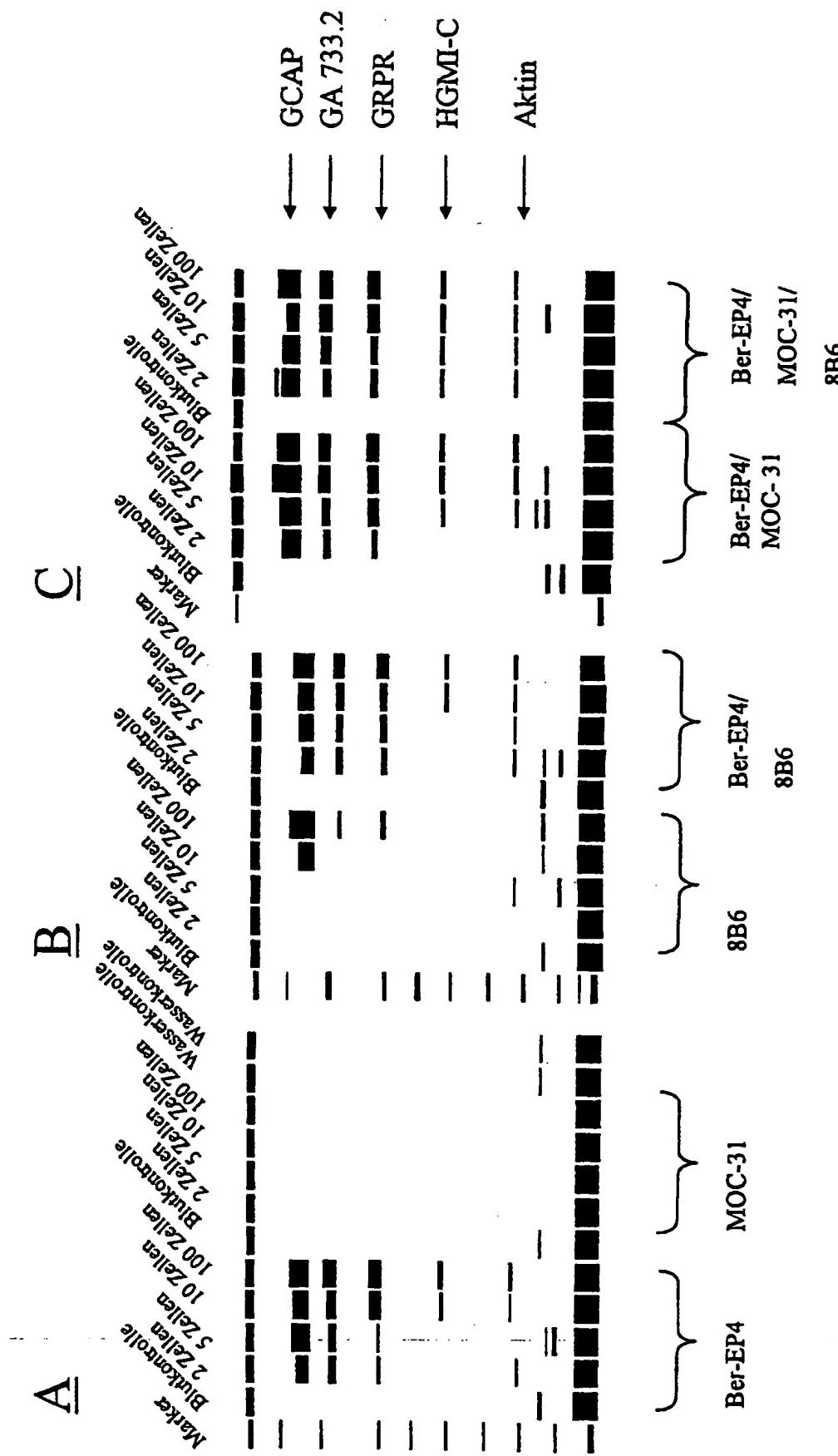
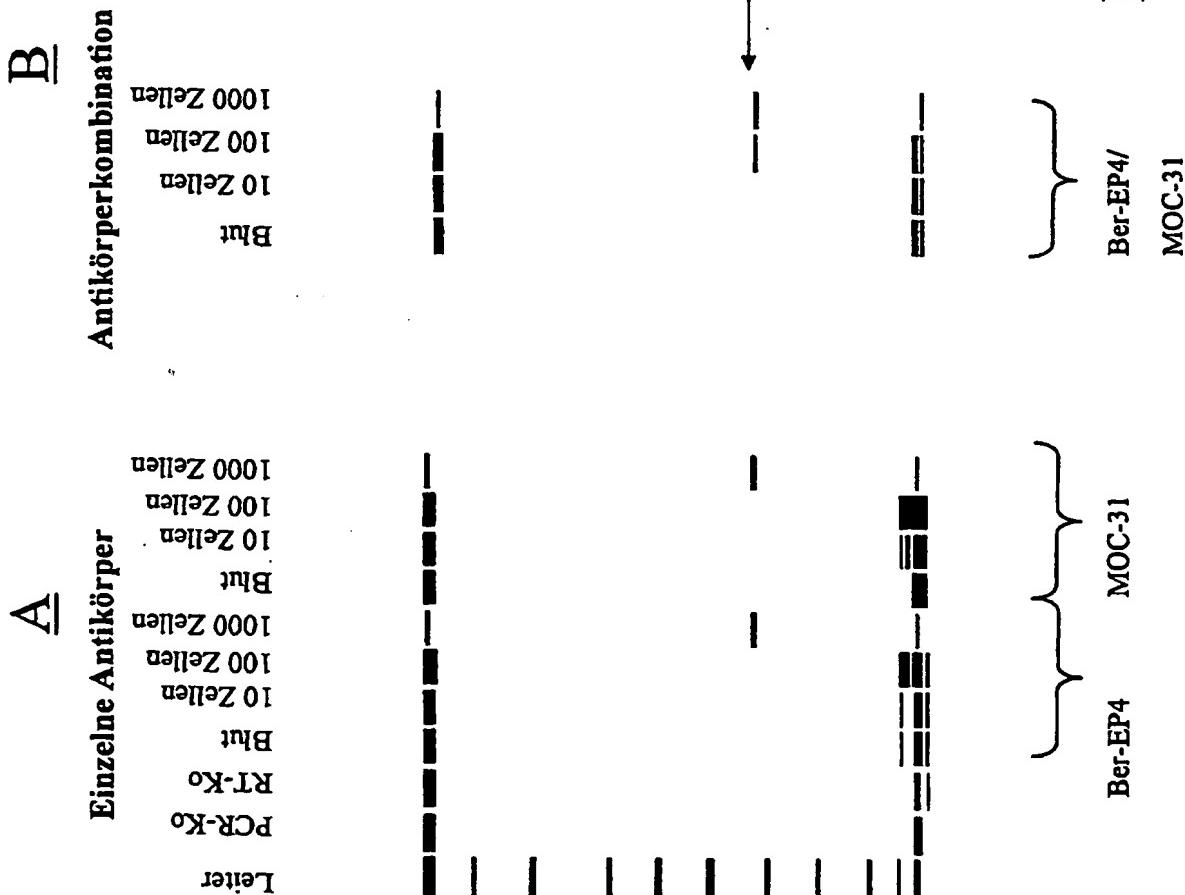


Fig. 3

**Fig. 4**

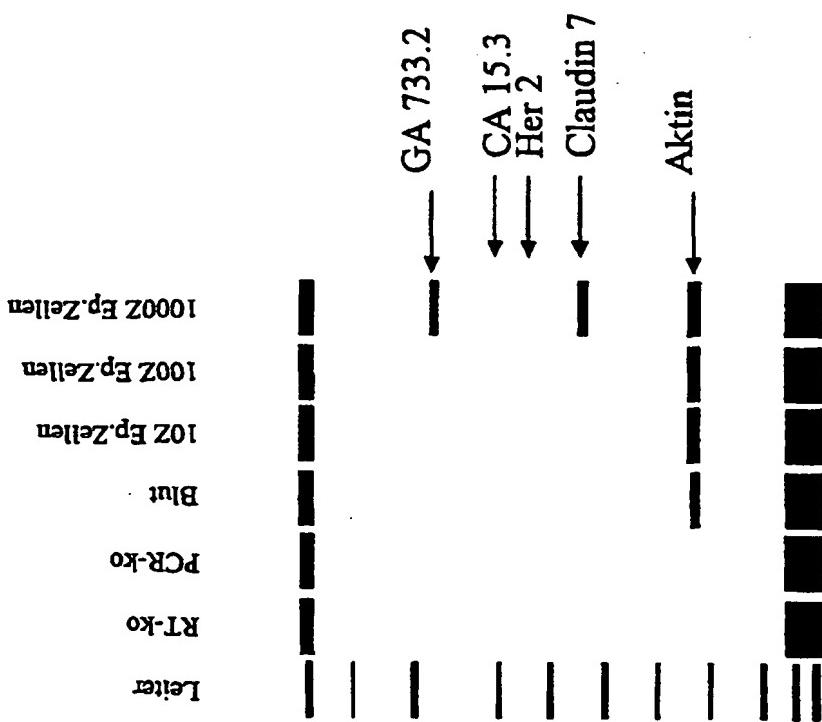


Fig. 5

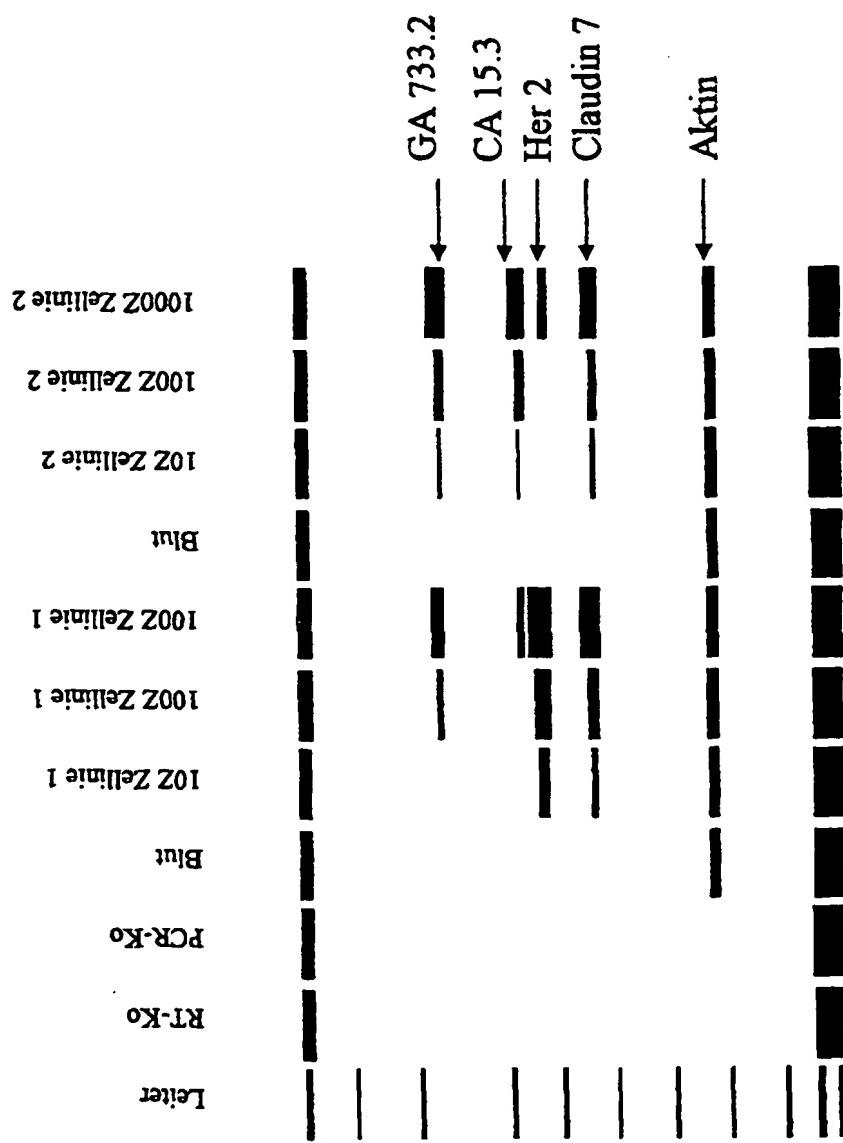


Fig. 6

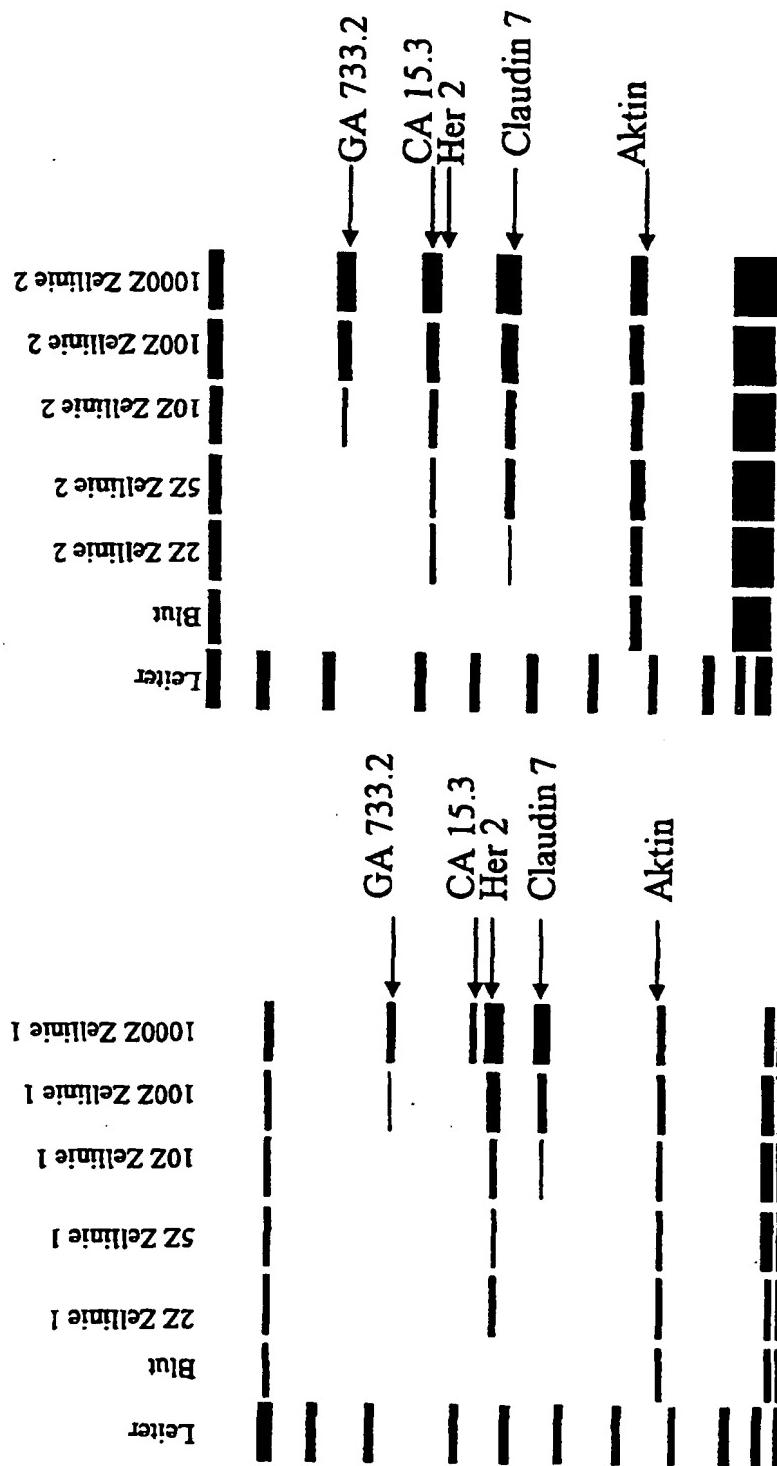
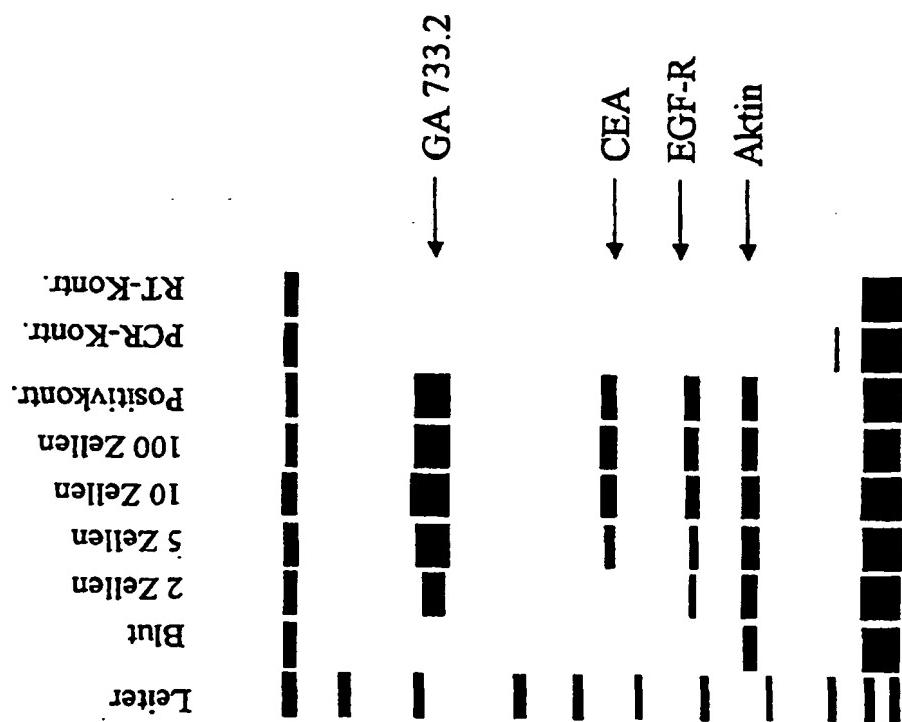
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Fig. 7

Fig. 8



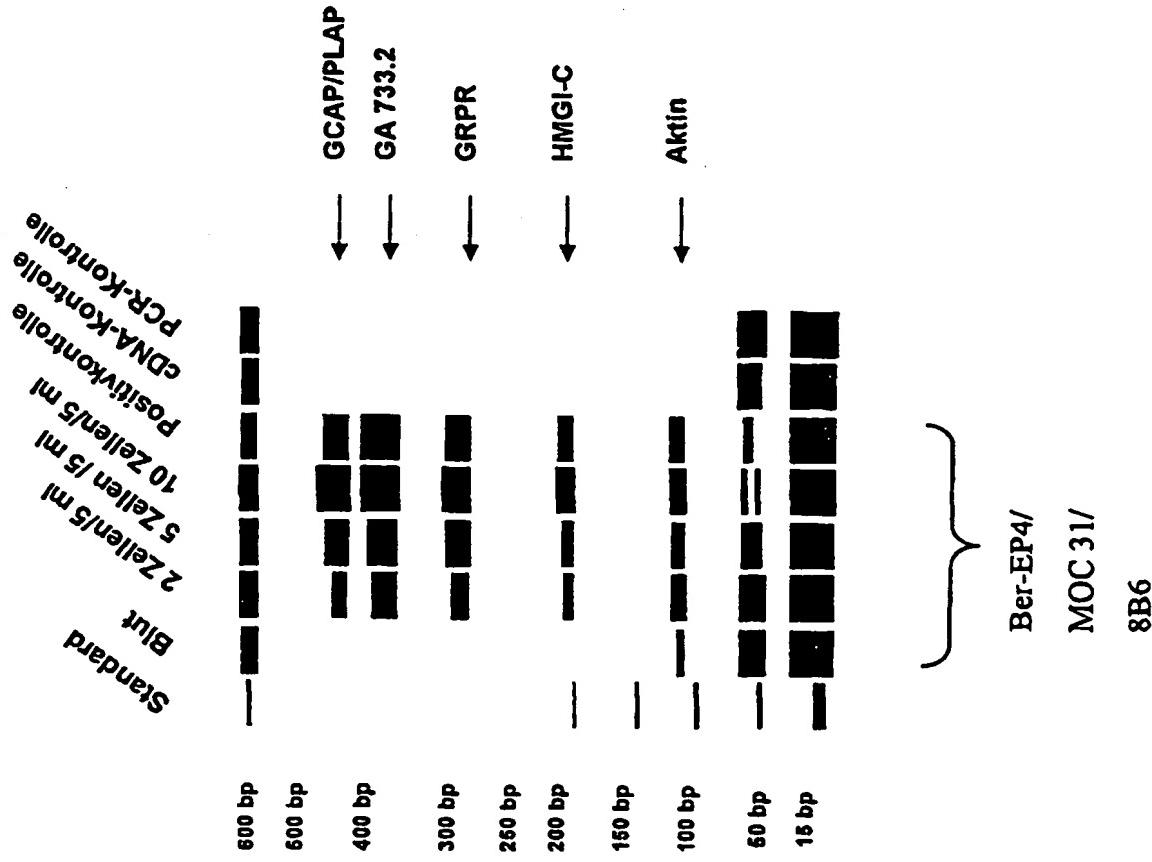


Fig. 9

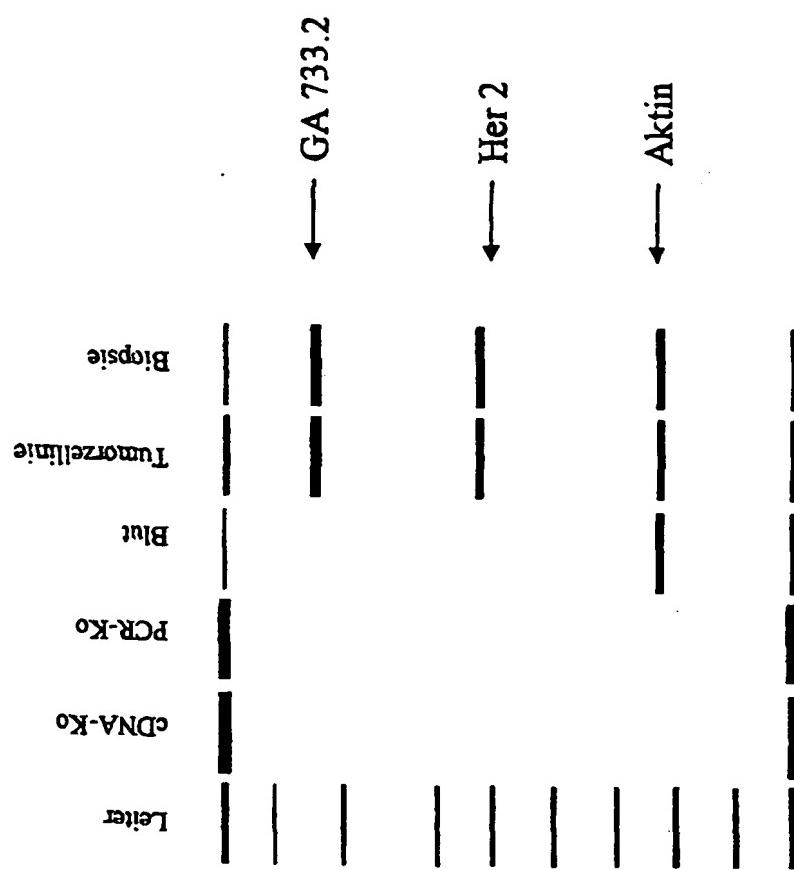


Fig. 10

(19)



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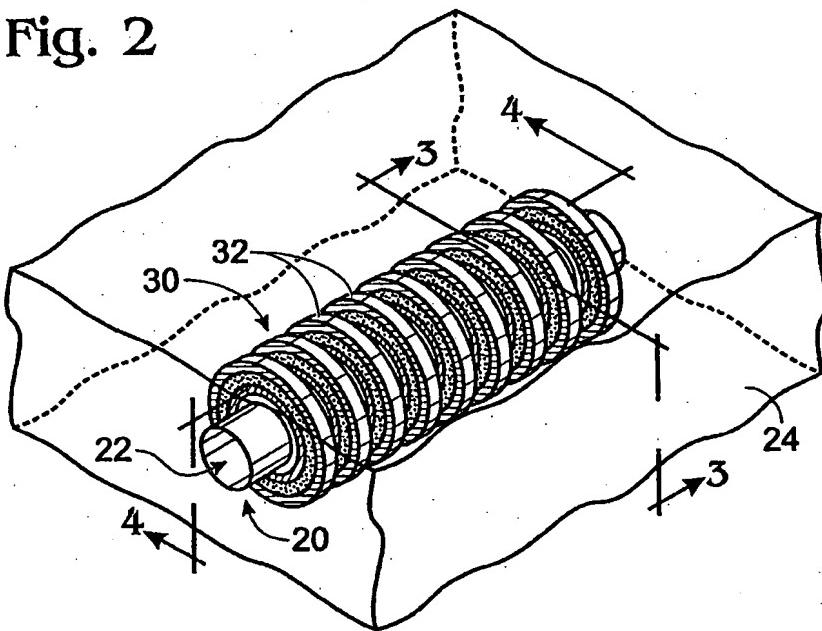
(54) Microfluidic pumping system

(57) A microfluidic device (10) including a fluidic pumping system (30) is provided. Some embodiments include a fluid-carrying channel (22), a plurality of acoustic pumping elements (32) arranged along the fluid-carrying channel, wherein the acoustic pumping elements (32) are configured to form an acoustic wave fo-

cused within the channel (22), and a controller (200) in electrical communication with the plurality of acoustic pumping elements (32), the controller (200) being configured to activate the acoustic pumping elements (32) in such a manner as to cause the acoustic wave to move along the channel (22) to move the fluid through the channel (22).

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Fig. 2



Description**BACKGROUND**

[0001] Recent advances in fluidic technology have led to the development of integrated chemical and biological analytical devices that place both electrical and fluidic systems on a single substrate. These devices sometimes are referred to as "laboratory-on-a-chip" devices, and may offer advantages over the use of larger, traditional analytical devices. For example, integrated analytical devices may consume smaller quantities of reagents and/or solvents, may occupy a smaller footprint in a laboratory, and/or may be easier to adapt for use in the field.

[0002] Fully or partially integrated chemical and biological analytical systems typically include a microfluidic network for moving fluids through the system. The term "microfluidic" typically refers to systems and processes for moving fluids through very small channels, for example, with micron-scale diameters. A microfluidic network may include a wide variety of components, including, but not limited to, valves for controlling access to fluid channels, mixers for mixing reaction components and/or carrier fluids, and pumps for moving fluids through the network.

[0003] Various types of pumps are known for use in microfluidics systems. For example, some microfluidics systems utilize mechanical pumps that move fluids through the system via mechanically created pressure differentials. However, such pumping devices may be difficult to fabricate, and also may be damaged by impurities in the sample. Other microfluidics systems may utilize electroosmotic pumping devices, in which an electric field is used to drive a polar fluid through a channel. However, these systems may utilize a high voltage (on the order of kilovolts) to drive movement of the fluid, and may be sensitive to impurities that adsorb to the wall of the channel. Furthermore, electroosmotic pumping devices may not be able to pump effectively nonpolar or only slightly polar solvents.

SUMMARY

[0004] Some embodiments of the present invention provide a microfluidic device including a fluidic pumping system. The fluidic pumping system includes a fluid-carrying channel, a plurality of acoustic pumping elements arranged along the fluid-carrying channel, wherein the acoustic pumping elements are configured to form an acoustic wave focused within the channel, and a controller in electrical communication with the plurality of acoustic pumping elements, the controller being configured to activate the acoustic pumping elements in such a manner as to cause the acoustic wave to move along the channel to move the fluid through the channel.

BRIEF DESCRIPTION OF THE DRAWINGS**[0005]**

- 5 Fig. 1 is a block diagram of an integrated analytical device according to an embodiment of the present invention.
10 Fig. 2 is an isometric view of a pumping system of the microfluidic network of the embodiment of Fig. 1.
15 Fig. 3 is a front sectional view of a pumping element taken along line 3-3 of Fig. 2.
20 Fig. 4 is a side sectional view of a plurality of pumping elements taken along line 4-4 of Fig. 2.
25 Fig. 5 is an isometric view of a pumping system according to a second embodiment of the present invention.
Fig. 6 is a front sectional view of a pumping element taken along line 6-6 of Fig. 5.
Fig. 7 is a side sectional view of a plurality of pumping elements taken along line 7-7 of Fig. 5.
Fig. 8 is a block diagram of an exemplary control system suitable for use with a pumping system according to the present invention.

DETAILED DESCRIPTION

[0006] Fig. 1 shows, generally at 10, a simplified block diagram of a microfluidic device according to an embodiment of the present invention. Microfluidic device 10 includes an input 12, a microfluidic network 14, and an output 16.

[0007] Input 12 is configured to serve as an interface between microfluidic network 14 and macroscopic components positioned upstream of the microfluidic network in an overall process flow, and may be configured to accept the introduction of one or more fluids into device 10. Any suitable fluid may be introduced into microfluidic device 10 via input 12. Examples include, but are not limited to, biological or chemical samples contained within a liquid- or gas-phase carrier, and solvents, reagents, and other chemical components.

[0008] Input 12 may include a single input for receiving a single analytical sample mixture, or may include multiple individual inputs for receiving a plurality of substances. For example, input 12 may include a plurality of sample inputs that allow multiple samples to be introduced simultaneously to microfluidic device 10 for sequential or parallel processing. Input 12 may also include a plurality of reagent inputs configured to introduce one or more reagents for reaction with a sample. Furthermore, input 12 may include electrical inputs configured to accept electrical signals for controlling and/or powering the various components of microfluidic network 14.

[0009] Likewise, output 16 is configured to serve as an interface between microfluidic network 14 and macroscopic components that are positioned downstream

of the microfluidic network in an overall process flow. Output 16 may include both electrical and fluidic outputs. For example, output 16 may include a fluidic output configured to deposit waste fluids into a waste receptacle, or to route fluids into analytical instruments after processing within microfluidic network 14. Output 16 may also include electrical outputs configured to output electrical signals. Examples of electrical signals that may be output from microfluidic network 14 include, but are not limited to, raw or processed data signals from an integrated sensor and/or circuit formed within microfluidic network 14.

[0010] Microfluidic network 14 may have any desired selection and arrangement of microfluidic components suitable for performing a selected task. Exemplary components that may be included in microfluidic network 14 include, but are not limited to, mixers, storage chambers, separation columns, and channels, valves and pumping systems for connecting the various components.

[0011] Fig. 2 shows, generally at 20, an exemplary channel structure and, generally at 30, a pumping system from microfluidic network 14 for moving a fluid through the microfluidic network. Channel structure 20 includes a fluid-carrying channel 22 formed within a substrate 24 that is configured to carry a fluid between components within microfluidic network 14. Channel structure 20 may also include a corrosion-resistant material 26 (Fig. 3) to protect the inner surfaces of the channel from corrosive fluids. Alternatively, channel structure may not include material 26 on its inner surface, if the fluids being pumped are not significantly corrosive to the materials used to form channel structure 20. In these embodiments, the inner surface of the channel may be at least partially formed by pumping elements 32 of pumping system 30, which are described in more detail below. Pumping system 30 is configured to move fluids through channel 22 via the formation of focused acoustic waves within the channel. Pumping system 30 may also be configured to move the acoustic waves along the length of channel 22 to create a peristaltic pumping effect. To form the acoustic waves, pumping system 30 includes a plurality of individual pumping elements 32 disposed along the length of channel 22. While Fig. 2 shows nine pumping elements 32 spaced evenly along the length of channel 22, it will be appreciated that any suitable number of pumping elements may be disposed along the length of the channel. Furthermore, while the depicted pumping elements 32 are evenly spaced along the length of channel 22, the pumping elements may also be spaced in any other manner along the length of the channel suitable to cause an acoustic wave to be focused at a desired location within the channel.

[0012] Pumping elements 32 are shown in more detail in Figs. 3-4. Each pumping element includes an inner electrode 34, an outer electrode 36, and a piezoelectric element 38 positioned between the inner and outer electrodes. The depicted pumping elements have a ring-

shaped configuration, and concentrically surround channel 22. The application of a voltage pulse across piezoelectric element 38 via inner electrode 34 and outer electrode 36 causes the piezoelectric element to change physical dimensions, which thus creates a ring-shaped-acoustic wave. The pulse may be of a constant voltage, or may be of a periodic voltage. Where a pulse of a periodic voltage is used to activate pumping elements 32, the acoustic energy from one or more pumping elements 32 may constructively add at a selected location within channel 22, herein after referred to as the "focal region," to form a focused acoustic wave within the channel via acoustic Fresnel diffraction. It will be appreciated that, where protective material 26 is used to coat the interior of channel 22, the layer of material 26 may be segmented in some manner to form expansion joints to allow the material to withstand the contractions and expansions of piezoelectric elements 38.

[0013] When an acoustic wave formed by one or more pumping element 32 is focused in channel 22, the pressure at the focal region is increased relative to the areas within channel 22 adjacent the focal region. This increase in local pressure at the focal region may drive a fluid within channel 22 away from the focal region. In this manner, a fluid may be moved along channel 22 by applying a pattern of pulses to the plurality of pumping elements 32 in a progressive manner along the length of channel 22.

[0014] The plurality of pumping elements 32 may be activated in any number of different patterns and/or manners to form a focused acoustic wave within channel 22. Where it is desired to position the focal region at the center of channel 22, a plurality of elements, each with a spacing of a multiple of one acoustic wavelength from the desired focal region, may be simultaneously activated. For example, in the embodiment depicted in Fig. 4, elements 32', 32" and 32''' may be simultaneously activated, and if they are spaced such that they are located multiples of one wavelength from a focal region within channel 22 (for example, in the plane of element 32'), a focused acoustic wave will be formed in the channel at the focal region. Thus, where the sum of the radius of channel 22, the thickness of corrosion-proof material 26, and the thickness of inner electrode 34 is approximately 10 microns, and where channel 22 is configured to contain pure water (velocity of longitudinal waves in pure water is approximately 1480 m/s), then the application of a pulse containing a signal with a frequency of approximately 148 MHz may cause the production of a primary acoustic wave with a wavelength of approximately 10 microns, and thus having a focal region in the plane of the element that produced the primary wave. Other elements spaced multiples of this wavelength from the focal region may be simultaneously activated to add constructively with the primary wave, and thus to form a focused wave at the focal region.

[0015] Likewise, the focal region may be located at any other desired point within channel 22, either along

the length of or radially between the center and side of, channel 22 by selecting suitable combinations of elements which are simultaneously activated according to the following relationship (for a "positive" Fresnel Half-Wave Band pattern having a concentric electrode pattern with an open center; other relationships may describe other electrode patterns):

$$\sqrt{r_n^2 + F^2} - F = \frac{n\lambda_l}{4}$$

where r_n is the inner radius of a selected piezoelectric element 38, F is the focal length of the selected piezoelectric element, λ_l is the wavelength of the acoustic wave formed by the selected piezoelectric element at a selected RF frequency, and $n = 1, 3, 5, \dots$. Alternatively, the location of the focal region within channel 22 may also be varied by varying the frequency of the RF power applied to the piezoelectric element 38.

[0016] Inner electrode 34, outer electrode 36 and piezoelectric element 38 may have any suitable dimensions. For example, inner electrode 34, outer electrode 36 and piezoelectric element 38 may each have a width (along the flow direction of channel 22), as narrow as 1-2 microns, as wide as 10-20 microns, or outside of these ranges.

[0017] Inner electrode 34, outer electrode 36 and piezoelectric element 36 may also have any desired thickness (along the radial direction of channel 22). For example, inner electrode 35 may have a thickness selected on the basis of how far the inner surface 40 of piezoelectric element 38 is to be located from the center of the channel to position the focal region in a desired location within channel 22. Furthermore, in some embodiments of the invention (described in more detail below), each pumping element 32 may include more than one piezoelectric element. In this situation, the radial thickness of each piezoelectric element and associated electrode pair may be chosen to provide resonance at a more desirable resonant frequency (which is determined by the materials used to form each pumping element, and the thickness of the materials).

[0018] Inner electrode 34 and outer electrode 36 may be made from any suitable material. For example, inner electrode 34 and outer electrode 36 may be made from an electrically conductive material selected for its compatibility with a desired manufacturing process. Where inner electrode 34 and outer electrode 36 are made from a material that is resistant to the fluids that are to flow through channel 22, corrosion-resistant layer 36 may be omitted if desired. Examples of suitable materials for inner electrode 34 and outer electrode 36 include, but are not limited to, aluminum, copper, gold, and other electrically conductive materials.

[0019] Likewise, piezoelectric element 38 may be made from any suitable piezoelectric material. Examples of suitable materials include, but are not limited to,

zinc oxide, quartz, lithium niobate, and lithium titanate. The piezoelectric material used to construct piezoelectric element 38 may be deposited or otherwise formed in any suitable orientation. For example, where piezoelectric element 38 is formed from zinc oxide, the [111] orientation of the zinc oxide may be directed toward the center of channel 22 to direct the acoustic wave toward the center of the channel in the plane of pumping element 32.

- 10 [0020] Each pumping element 32 may be spaced from adjacent pumping elements 32 by any suitable distance. For example, the spacing of pumping elements 32 may be configured cause the acoustic energy emitted by selected elements to constructively interfere at a desired location by acoustic fresnel focusing. Where RF energy is used to activate pumping elements 32, exemplary distances include, but are not limited to, those in the range of two to six microns. The distance separating adjacent pumping elements 32 may possibly depend upon the desired mode of operation of the pumping elements. For example, adjacent pumping elements 32 may be configured to have different focal points in different locations within channel 22. In this manner, multiple adjacent pumping elements 32 may be activated simultaneously to create a progressively pinched focal region in the direction of fluid flow in the channel by effectively forming a pressure gradient. Likewise, multiple pumping elements 32 may be activated simultaneously to create a larger area of increased pressure within channel 32.
- 15 [0021] Figs. 5-7 show, generally at 130, a second embodiment of a pumping system suitable for use in microfluidic network 14. Pumping system 130 is similar to pumping system 30 in that a series of piezoelectric pumping elements are arranged along the length of a fluid-carrying channel 122 to move a fluid through the channel. Each pumping element 132 may be activated to form an acoustic pressure wave focused at a selected location within the interior of channel 122, and more than one adjacent pumping element may be activated simultaneously as desired to obtain the desired focused acoustic wave. Also, the pattern of activated pumping elements 132 may be shifted along the length of channel 122 to cause the acoustic pressure wave to move along the channel, and thus to move a fluid through the channel via a peristaltic pumping effect.
- 20 [0022] However, unlike pumping elements 32, pumping elements 132 each include two concentric piezoelectric elements. Referring to Figs. 6-7, each pumping element 132 includes an inner electrode 134, an intermediate electrode 136, an inner piezoelectric element 138 disposed between the inner electrode and intermediate electrode, an outer electrode 140, and an outer piezoelectric element 142 disposed between the intermediate electrode and the outer electrode.
- 25 [0023] Pumping system 130 may be operated in different manners to create different pumping effects. For example, a single voltage pulse may be applied simul-

taneously across inner piezoelectric element 138 and outer piezoelectric element 142. Where the geometries of inner piezoelectric element 138 and outer piezoelectric element 142 are configured to cause the pressure waves from each piezoelectric element to constructively interfere at the same focal region within channel 122, a more powerful pressure wave may be produced relative to the single piezoelectric configuration of pumping elements 32. Alternatively, inner piezoelectric element 138 and outer piezoelectric element 142 may be configured to be individually controllable.

[0024] Each of inner electrode 134, intermediate electrode 136, inner piezoelectric element 138, outer electrode 140 and outer piezoelectric element 142 may have any suitable dimensions. For example, the spacing between adjacent pumping elements, and the width of each individual pumping element, may have values within the ranges discussed above for pumping elements 32.

[0025] Likewise, inner electrode 134, intermediate electrode 136, inner piezoelectric element 138, outer electrode 140 and outer piezoelectric element 142 each may have any suitable thickness in the radial direction. To avoid problems with destructive interference, the thickness of each of these elements may be selected to set the distances from the inner surface 144 of inner piezoelectric element 138, and from the inner surface 146 of outer piezoelectric element 142, a multiple of the wavelength of the acoustic wave generated by the piezoelectric elements. Because outer piezoelectric element 142 is located farther from the inner wall of channel 122, the different velocities at which the acoustic wave generated by the outer piezoelectric element may travel through the different piezoelectric and electrode layers before reaching channel 120 may need to be taken into account when calculating the location of the focal region.

[0026] While the embodiments of Figs. 2-7 depict pumping elements 32 and 132 having one or two concentric piezoelectric elements, respectively, a pumping element according to the present invention may have three, or even more, concentric piezoelectric elements if desired. Also, while the individual pumping elements are shown as being generally circular in shape, it will be appreciated that the pumping elements may have any other suitable shape, including but not limited to, rectangular or triangular. Furthermore, while the piezoelectric elements of each of the depicted embodiments concentrically surround the associated fluid-carrying channel, the piezoelectric elements may have any other suitable geometric relationship to the fluid-carrying channel and to each other.

[0027] Fig. 8 shows, generally at 200, a block diagram of an exemplary control system suitable for use with a pumping system according to the present invention. While control system 200 is described below in the context of pumping system 30, it will be appreciated that the discussion applies equally to pumping system 130, or

any other suitable pumping system. Control system 200 is configured to create a focused acoustic pressure wave of a fixed profile within channel 22 by activating selected pumping elements 32, and also to move the focused pressure wave along the length of channel 22 to create a peristaltic pumping effect.

[0028] Pumping system 200 includes various components that cooperate to create the focused pressure wave and peristaltic pumping effect. First, pumping system 200 includes a programmable rate oscillator, indicated generally at 202. Programmable rate oscillator 202 outputs a pulse train 203 at a selected rate, as described in more detail below, to set a pumping rate. The rate may be user-selected, or specified by system programming. Second, pumping system 200 includes a pattern holding register 204 for holding data representing a selected profile for the focused pressure wave, and for shifting the selected profile along the length of channel 22 at the rate determined by programmable rate oscillator 202 to create the peristaltic pumping effect.

[0029] Programmable rate oscillator 202 includes a master oscillator 206 for creating a master clock pulse, and a programmable divider 208 for reducing the frequency of the clock pulse to a selected frequency. Programmable rate oscillator 202 also may include a rate holding register 210 that holds data representing the selected output frequency. Rate holding register 210 may be configured to accept input of a selected pumping rate from a user via pumping rate input device 212, or may contain data representing a fixed pumping rate.

[0030] Likewise, pattern holding register 204 may be connected to a pattern input 214 that allows a user to input a selected focal pattern or profile. The focal pattern contains data that sets the shape of the focal pattern to be formed in channel 22. The focal pattern may include any data suitable for forming a selected focal pattern. For example, the focal pattern may include data representing which piezoelectric elements 38 of the plurality of piezoelectric elements are to be simultaneously activated at any selected time.

[0031] Furthermore, in some embodiments of the invention, the location of the focal region of each piezoelectric element 38 may be selectively variable. The focal region of a selected piezoelectric element 38 may be varied in any suitable manner. For example, the frequency of the signal contained within the voltage pulse that activates the piezoelectric element may be varied, or different pumping elements may be activated to cause constructive interference at different locations, radial or lengthwise, within the channel. Where the frequency of the signal is varied, the focal pattern held within pattern holding register 204 may include data that indicates the frequency of the activation pulse to be sent to each piezoelectric element.

[0032] Pattern holding register 204 may also include a plurality of outputs 216 for providing signals to a set of piezoelectric drivers 218, indicating which piezoelectric elements are to be activated. Each output 216 is in

electrical communication with a corresponding piezoelectric driver 218, and each piezoelectric driver is in electrical communication with a corresponding piezoelectric element 38. Each piezoelectric driver 218 is also in electrical communication with a periodic signal source, such as an RF source 220, that outputs a periodic signal 221 of a selected frequency or frequencies. Activation of a selected piezoelectric driver 218 by pattern holding register 204 causes the piezoelectric driver to send a conditioned RF pulse 222 from RF source 220 to the corresponding piezoelectric element 38. This causes the corresponding piezoelectric element 38 to produce an acoustic pressure wave within channel 22. The pulse width of the pulses in pulse train 203 from programmable rate oscillator 202 typically sets the width of RF pulse 222 sent to the piezoelectric elements. Alternatively, piezoelectric drivers 218 may be configured to regulate this pulse width. While the frequency of pulse 222 is described herein as being in the radiofrequency spectrum, pulse 222 may have any other suitable frequency for forming an acoustic wave in a selected channel, depending upon the dimensions of the selected channel.

[0033] Pattern holding register 204 may also be configured to move the focal pattern along the length of channel 22. In this manner, the pressure wave formed by each pumping element 32 is moved along the interior of channel 22 to push a fluid through channel 22. Pattern holding register 204 may be configured to shift the focal pattern along the length of channel 22 in any suitable manner. For example, pattern holding register 204 may act as a shift register and move the signal at each output 216 of the pattern holding register to the next adjacent output with each pulse from programmable rate oscillator 202. Alternatively, pattern holding register 204 may be configured to shift the signal at each output 216 by more than one position with each pulse from programmable rate oscillator 202 if desired.

[0034] Furthermore, if a continuous pumping action is desired, control system 200 may be configured to shift the selected focal pattern repeatedly along the length of channel 22. The repeated shifting of the selected focal pattern along the length of channel 22 may be accomplished in any suitable manner. In the depicted embodiment, the signal at the last output 216 of pattern holding register 204 may be fed back into the pattern holding register, as indicated at 224, to be applied to a more-upstream output 216. In this configuration, as each output signal of the focal pattern is shifted to the most-downstream output, the output signal is automatically fed back to an earlier output to begin a new movement along channel 22. In this manner, the focal pattern may be continuously recirculated to an upstream point of channel 22, and thus repeatedly shifted along the length of the channel to create a continuous pumping effect. It will be appreciated that the pumping direction of pumping system 32 may be reversed simply by reversing the direction pattern holding register 204 moves the focal pattern moves along channel 22.

[0035] Although the present disclosure includes specific embodiments, specific embodiments are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the present disclosure includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. These claims may refer to "an" element or "a first" element or the equivalent thereof. Such claims should be understood to include incorporation of one or more such elements, neither requiring nor excluding two or more such elements. Other combinations and subcombinations of features, functions, elements, and/or properties may be claimed through amendment of the present claims or through presentation of new claims in this or a related application. Such claims, whether broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the present disclosure.

Claims

- 25 1. An integrated microfluidic device (10) having a fluidic pumping system (30) for transporting a fluid through the device, the fluidic pumping system (30) comprising: a fluid-carrying channel (22) formed in the device (10); a plurality of acoustic pumping elements (32) disposed along the fluid-carrying channel (22), wherein the acoustic pumping elements (32) are configured to form an acoustic pressure wave focused within the channel (22); and a controller (200) in electrical communication with the plurality of acoustic pumping elements (32), the controller (200) being configured to activate the acoustic pumping elements (32) in such a manner as to cause the acoustic wave (22) to move along the channel (22) to move the fluid through the channel (22).
- 30 2. The microfluidic device (10) of claim 1, wherein the controller (200) is configured to activate the acoustic pumping elements (32) by supplying pulses of RF power to the acoustic pumping elements (32).
- 35 3. The microfluidic device (10) of claim 1, wherein each acoustic pumping element (32) of the plurality of acoustic pumping elements (32) includes a piezoelectric element (32) having a layer of piezoelectric material (38) disposed between a pair of electrodes (34), (36).
- 40 4. The microfluidic device (10) of claim 3, wherein each piezoelectric element (32) concentrically surrounds the channel (22).
- 45 5. The microfluidic device (10) of claim 1, wherein the controller (200) is configured to move the acoustic wave (22) along the channel (22) to move the fluid through the channel (22).
- 50 6. The microfluidic device (10) of claim 1, wherein the controller (200) is configured to move the acoustic wave (22) along the channel (22) to move the fluid through the channel (22).
- 55 7. The microfluidic device (10) of claim 1, wherein the controller (200) is configured to move the acoustic wave (22) along the channel (22) to move the fluid through the channel (22).

5. The microfluidic device (10) of claim 3, wherein each piezoelectric element (132) includes a plurality of layers of piezoelectric material (138), (142), each layer of piezoelectric material (138), (142) being disposed between a complementary pair of electrodes (134), (136), (140). 5
6. The microfluidic device (10) of claim 3, the channel (22) having a radial inner dimension, wherein selected piezoelectric elements (32) may be activated to form the focused acoustic wave at a focal region at a location within the channel (22), wherein the location of the focal region is configured to be positionally variable across the radial inner dimension of the channel (22), wherein a plurality of piezoelectric elements (32) are simultaneously activated to create a plurality of corresponding acoustic waves that constructively interfere at the focal region, and wherein the location of the focal region is varied by varying which of the plurality of piezoelectric elements (32) are simultaneously activated. 10 15
7. The microfluidic device (10) of claim 1, wherein the controller (200) includes a pattern holding register (204) in electrical communication with the plurality of acoustic elements (32), the pattern holding register (204) being configured to store data representing a preselected focal pattern that indicates the piezoelectric elements to be activated at a selected time, and wherein the pattern holding register (204) is configured to selectively shift the focal pattern along the plurality of acoustic pumping elements (32) in a progressive manner to create a peristaltic pumping effect. 20 25 30 35
8. A microfluidic device (10) including a pumping system (30) for moving a fluid through the device, the pumping system (30) comprising: a channel (22) formed in the device (10), wherein the channel (22) is configured to accommodate passage of the fluid; and a pumping element (32) associated with the channel (22), wherein the pumping element (32) includes a plurality of concentric piezoelectric elements (32) configured to produce a focused acoustic wave within the channel (22) to move the fluid through the channel (22). 40 45
9. A microfluidic device (10) for the analysis of a sample in a fluid, the microfluidic device (10) including: a microfluidic network (14) for transporting the fluid through the device (10), the microfluidic network (14) including a channel (22) configured to accommodate passage of the fluid; and means (32) for pumping the fluid through the channel (22). 50 55
10. A method of transporting a fluid in a device (10), the device (10) including a channel (22) configured to accommodate passage of the fluid and a plurality of acoustic pumping elements (32) disposed along the channel, each acoustic pumping element (32) having a focal region positioned within the channel (32), the method comprising: applying a focal pattern to the plurality of acoustic pumping elements (32), the focal pattern defining a selected subset of the plurality of acoustic pumping elements (32) to be activated simultaneously to create a pressure wave within the channel (22); and shifting the focal pattern by at least one acoustic pumping element (32) to move the pressure wave along the channel (22).

Fig. 1

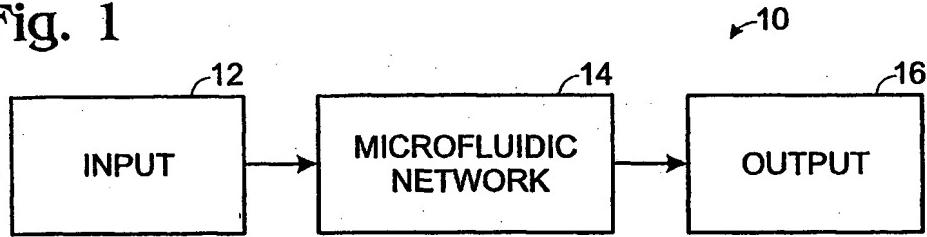


Fig. 8

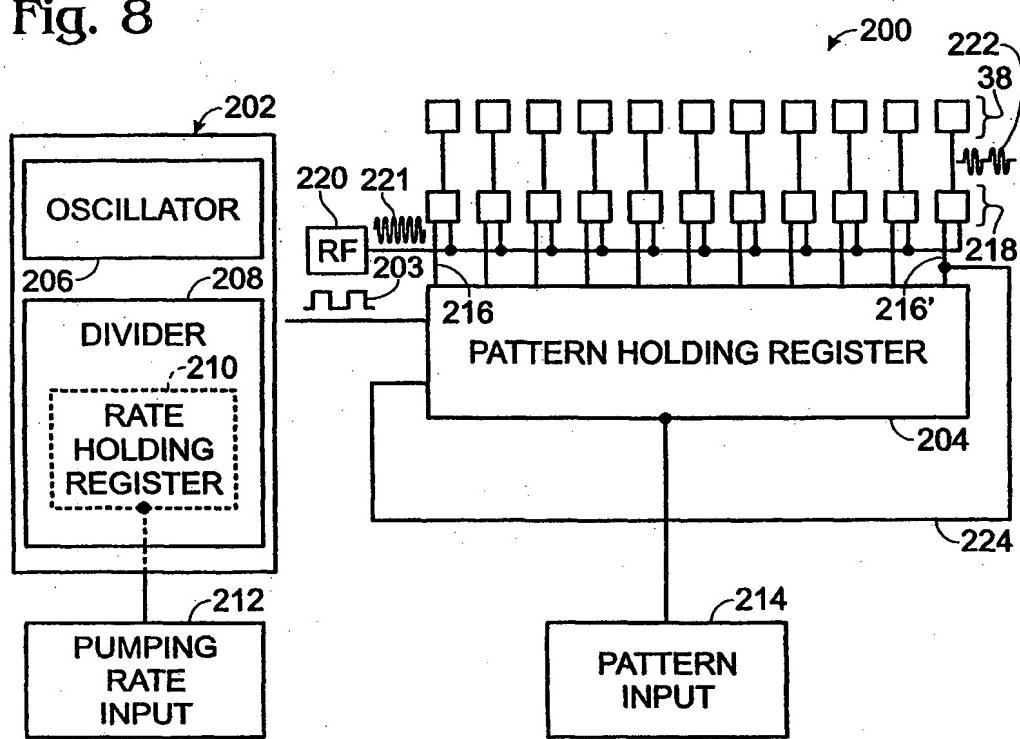


Fig. 2

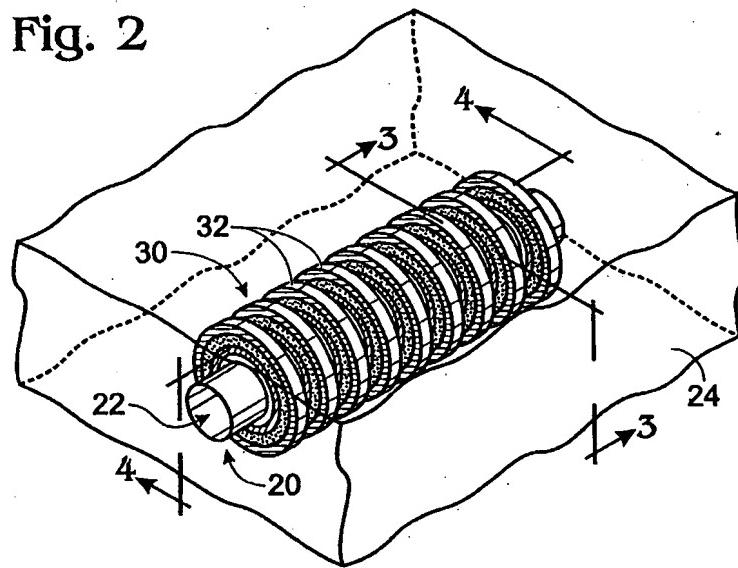


Fig. 3

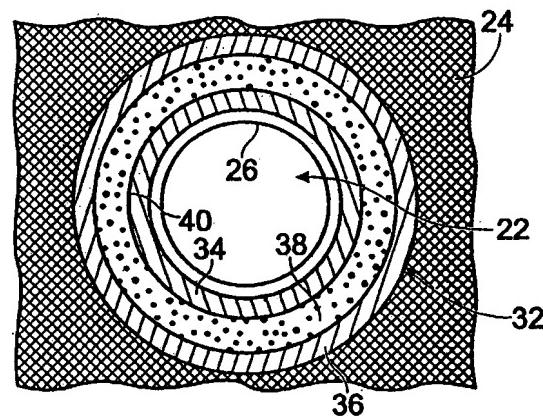


Fig. 4

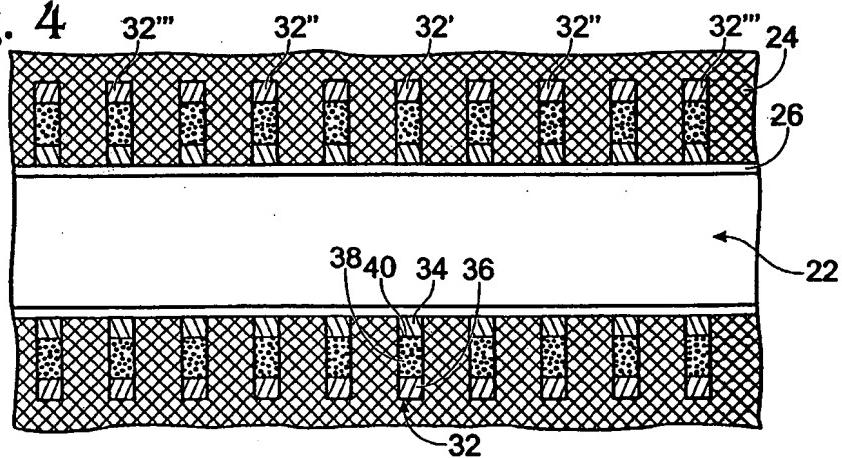


Fig. 5

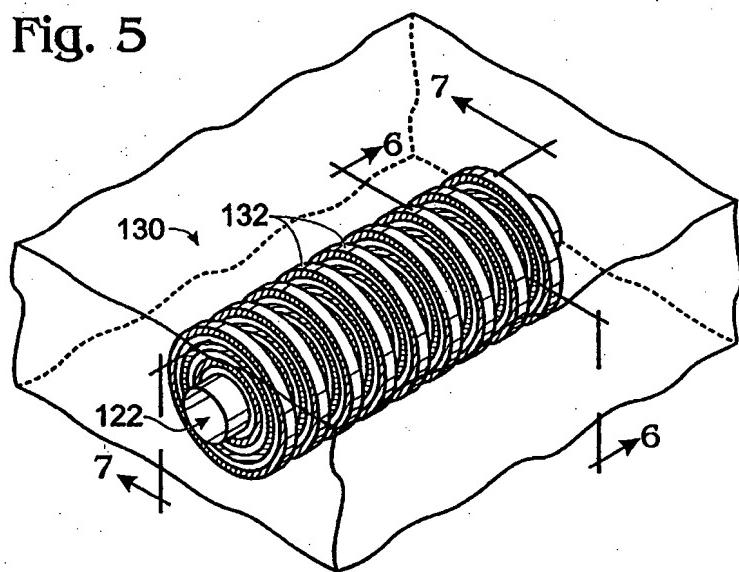


Fig. 6

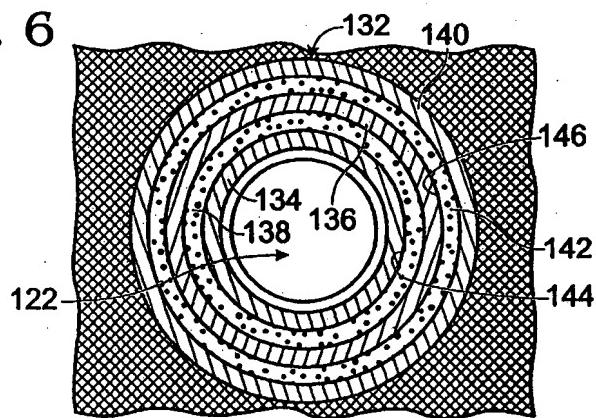
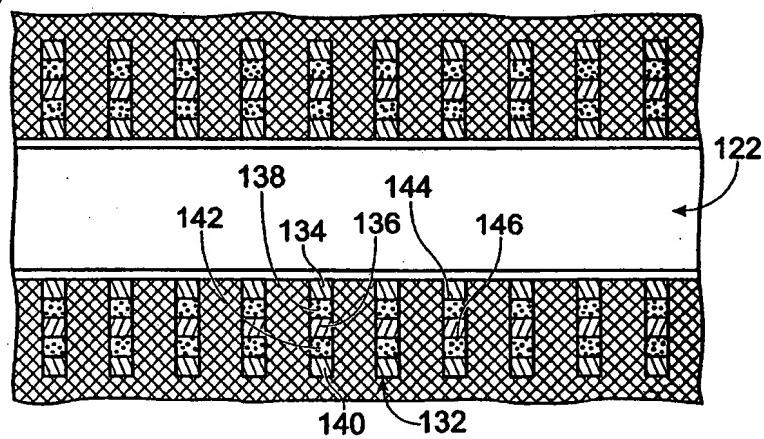


Fig. 7





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 03 25 6805

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|---|--|--|---|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.7) |
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| X | WO 02 064821 A (ADVALYTIX AG ;GAUER CHRISTOPH (DE)) 22 August 2002 (2002-08-22) * page 13, line 21 - page 15, line 35 * --- | 1, 9, 10 | |
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| <p>The present search report has been drawn up for all claims</p> | | | |
| Place of search | Date of completion of the search | Examiner | |
| MUNICH | 18 December 2003 | Tragoustis, M | |
| CATEGORY OF CITED DOCUMENTS | | | |
| X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document | | T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document | |
| EPO FORM 1503 03 82 (P01/01) | | | |

ANNEX TO THE EUROPEAN SEARCH REPORT
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EP 03 25 6805

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18-12-2003

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(54) BLOOD CELL SEPARATION SYSTEM

(57) A blood cell separating system is offered for precisely separating and concentrating rare fetal nucleated cells intermixed in the blood of a pregnant woman, to conveniently obtain test preparations capable of being used for prenatal chromosomal/genetic diagnosis. A blood cell separating system is characterized by comprising (1) a primary separating device for removing mainly non-nucleated erythrocytes, leukocytes and platelets from blood samples taken from a pregnant

woman to obtain a primary separated sample, (2) a secondary separating device for using a carbohydrate-lectin method to remove residual non-nucleated erythrocytes and leukocytes from the primary separated sample obtained by the primary separating device to obtain a secondary separated sample with concentrated fetal nucleated cells, and (3) preparing device for preparing the secondary separated sample obtained by the secondary separating device.

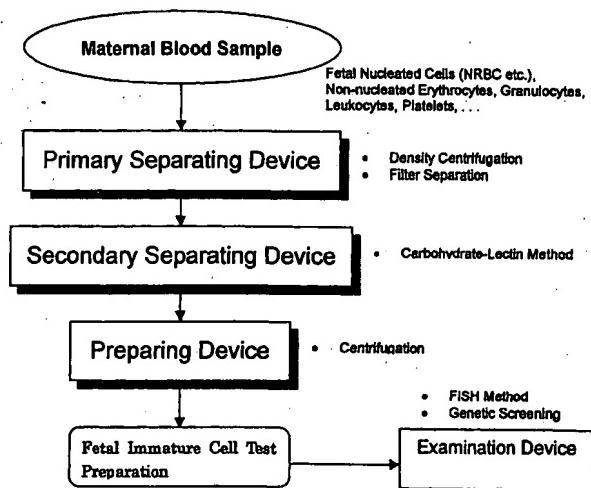


FIG. 1

Description**Technical Field**

5 [0001] The present invention relates to a system for the separation of blood cells using lectins, particularly to a system for efficiently removing non-nucleated erythrocytes and mature leukocytes, to separate and concentrate fetal nucleated erythroblasts, from samples containing nucleated erythroblasts which are fetal nucleated cells found in the maternal peripheral blood or umbilical blood of pregnant women, and further, a system for testing the chromosomes and genes thereof.

10

Background Art

15 [0002] In the field of genetic diagnosis, the development of methods for prenatal diagnosis which do not endanger the embryo, i.e. the fetus, has been long anticipated. The genetic diagnosis methods which are practiced clinically at present are invasive procedures such as amniocentesis, villus sampling and fetal blood collection, of which sampling of fetal cells by amniocentesis in particular allows for a positive diagnosis, but also carries a high risk of miscarriage at about 1/300.

20 [0003] Additionally, while methods for predicting fetal abnormalities from changes in maternal serum biochemical markers present in the maternal peripheral blood such as AFP (α -fetoprotein), hCG (chorionic gonadotropin), uE3 (uric Estriol 3, a type of estrogen secreted from the fetal adrenal glands during pregnancy and used in tests of fetal-placental function) and inhibin A (a type of gonadotropin) are desirable for being non-invasive, they are only methods for screening for the need to perform amniocentesis and cannot be relied upon to make any positive diagnoses.

25 [0004] On the other hand, clinical studies on the incompatibility of blood types between mother and child have made it dear that fetal cells can find their way into the maternal circulatory system. Thus, if intermixed fetal cells can be isolated from the maternal blood, and if they are nucleated cells, their DNA and chromosomes can be used to safely make positive fetal diagnoses. In 1969, Walknowska *et al.* reported that they had cultured maternal peripheral lymphocytes and discovered a 46XY karyotype in 21 of 30 women pregnant with male children. However, the work of extracting lymphocytes having a Y chromosome from an entire group of similar lymphocytes is extremely difficult, and attempts to separate and concentrate them by means of a nylon wool column or density centrifugation did not succeed in yielding a practical solution. Additionally, the presence of fetal cells remaining from past pregnancies was also considered to be a problem, and the development of a safe method of positive fetal diagnosis allowing for testing of a desired neonatal fetus did not progress any further.

30 [0005] In recent years, nucleated erythroblasts which are cells that have a short lifespan and are normally almost non-existent in the mother have garnered some attention as fetal nucleated cells present in the maternal blood, and research into the possibility of their use as cells to be used for diagnosis has become very active. However, the proportion of their presence in the maternal blood is extremely low at 1/10⁵ to 1/10⁷ of all nucleated cells, and just as with fetal cell diagnosis by lymphocytes, there are technical obstacles to achieving a process of separating and detecting a sufficient number of fetal nucleated erythroblasts from maternal nucleated cells such as leukocytes.

35 [0006] While attempts have been made to separate the erythroblasts by means of flow cytometry using transferin receptor antibodies or magnetic beads, the problem of specificity of antibodies has precluded the efficient detection of erythroblasts having Y chromosomes.

40 [0007] On the other hand, there are collection methods in which, instead of performing cell separation, the erythroblasts are identified morphologically and picked out one at a time by micromanipulation, but the process of discriminating erythroblasts from among masses of maternal nucleated cells requires considerable technical expertise and is extremely time-consuming. Thus, this method requires special equipment, and for example, can take several days to process a single specimen. At present, perhaps a single erythroblast will be detected from 1 cc of maternal peripheral blood. However, at least 30 fetal cells are considered to be necessary in order to establish a general and practical fetal testing method for testing genes and chromosomes in a statistically conclusive manner, which makes this sampling method unrealistic when bearing in mind that the quantity of blood which may be safely collected from a pregnant woman is at most 10 cc, so that even to this day, there is a strong demand for a method for quickly and conveniently separating and concentrating erythroblasts at a high yield.

45 [0008] The present inventors have been performing research with a focus on the specific interactions of carbohydrate chains, and have obtained a patent (Japanese Patent No. 3139957) on a cell culture matrix having a matrix surface of a dish or the like coated with glycoconjugate polymers having various carbohydrates as side chains, whereby lectins which have an affinity to these carbohydrate chains are selectively attached. Furthermore, they discovered that a separating method using lectins can be used to separate and concentrate not only erythroblasts, but also immature hematopoietic cells, thus making it possible to detect 10-30 fetal nucleated cells (International Publication WO00/58443).

[0009] When separating and recovering blood cells such as erythroblasts, the peripheral blood is generally first pre-treated using density centrifugation, but the erythroblasts were found to have been damaged during this pretreatment stage by density centrifugation. For example, the specific gravity of nucleated erythroblasts changes between about 1.077-1.095, and when a high-density fluid (such as Ficoll or the like) with a certain specific gravity is used, erythroblasts are sometimes detected from both the erythrocyte layer under the Ficoll layer and above the Ficoll layer, so that simple separation by density centrifugation can cause some of the erythroblasts to be lost. This is thought to be due to the uncertainty in the specific gravity of fetal erythroblasts which contain fetal hemoglobin and are about to denucleate.

5 [0010] Furthermore, in order to know of chromosome abnormalities and the like in nucleated erythroblasts separated and purified in this way, they must be inspected by the FISH (Fluorescence In Situ Hybridization) method or the like, but this gives rise to practical problems which need to be overcome during the process of preparing specimens appropriate for such testing.

10 [0011] Therefore, in order to completely resolve the above-described problems, the present invention has the object of providing a new blood cell separating system capable of being offered for clinical practice, based on precision separation and recovery of nucleated erythrocytes (NRBC) using lectins. That is, the present invention offers a system which considerably prevents the loss of erythroblasts during the pretreatment step while using lectins to precisely separate the rare fetal nucleated cells from the maternal cells in a maternal blood sample of a limited quantity, thereby to separate, concentrate and recover the desired immature fetal cells or NRBC's selectively and at a high yield, and to offer a method of producing a test preparation containing fetal cells separated and recovered using such a system.

15

20 **Summary of the Invention**

[0012] **The blood separating system of the present invention**

(1) comprises a primary (crude) separating device for removing mainly non-nucleated erythrocytes, leukocytes and platelets from a blood sample taken from a pregnant woman to obtain a primary (crude) separated sample, said primary separating device separating and removing mainly leukocytes which are nucleated cells, non-nucleated erythrocytes, platelets and the like under conditions which highly prevent loss of fetal nucleated cells among the various types of blood cells included in a maternal blood sample;

25 (2) comprises a secondary (precision) separating device for removing residual non-nucleated erythrocytes and leukocytes from a primary separated sample obtained from said primary separating device, to obtain thereby a secondary (precision) separated sample with concentrated fetal nucleated cells, the secondary separating device involving incubation of said primary separated sample, under conditions which inactivate the cells, along with a predetermined concentration of lectins, on a substrate having glycoconjugate polymers affixed to the surface thereof, thereby selectively binding fetal nucleated cells contained in said primary separated sample with said lectins

30 (3) to thereby concentrate and attach them to said substrate by means of a lectin-carbohydrate interaction to form a secondary separated sample; and

35 (4) comprises a preparing device for preparing a secondary separated sample obtained from said secondary separating device, the preparing device involving centrifugation, under predetermined conditions, of said substrate on which said fetal nucleated cells have been concentrated and attached, to thereby obtain a test preparation wherein fetal nucleated cells from maternal blood are present in a state advantageous to genetic/chromosomal testing by the FISH method or the like, at levels which allow for a positive diagnosis.

40

[0013] The blood separating system of the present invention should preferably further comprise a testing device for using a test preparation obtained from the aforementioned preparing device to test the chromosomes and/or genes in fetal nucleated cells contained in the test preparation.

45

[0014] Furthermore, the present invention also offers a method of producing a test preparation for a prenatal fetal diagnosis using the above-described blood cell separating system, as well as a test preparation produced by this production method, wherein immature fetal cells are present in a state advantageous to genetic/chromosomal testing by the FISH method or the like, at levels which allow for a positive diagnosis.

50

Brief Description of the Drawings

[0015]

55 Fig. 1 is a block diagram showing an example of a blood cell separating system of the present invention.
Fig. 2 is a microscopic (X200) photograph showing blood cells immobilized on a glycoconjugate-polymer-coated substrate from maternal blood using 300 µg/ml of lectin (SBA).
Fig. 3 is a microscopic (X200) photograph showing blood cells immobilized on a glycoconjugate-polymer-coated

substrate from maternal blood using 8 µg/ml of lectin (SBA).

Fig. 4 is a microscopic (X1000) photograph showing the immobilized cells shown in Fig. 3.

Fig. 5 is a cross-section diagram showing an example of a substrate usable in blood cell separating system of the present invention.

5

Descriptions of Preferred Embodiments

[0016] Herebelow, the present invention shall be described in detail.

[0017] First, a blood sample to be used in the system and method of the present invention is taken from a pregnant woman. As blood collecting means, a blood collecting tool which is generally used, such as a syringe, vacuum blood collecting tube or a blood bag may be used. The blood to be taken may be any type of blood including venous blood, umbilical blood, intervillous blood from the placenta or myeloid fluid, but in order to limit the intrusion into the maternal body as much as possible, peripheral venous blood is the most preferable. While there is no particular restriction on the quantity of the blood sample, it should generally be about 1-10 ml in view of the problems posed by invasion of the maternal body. In order to prevent the taken blood sample from coagulating, EDTA, heparin, CPD/ACC or the like should preferably be added. In order to obtain meaningful results, it would be preferable to use a fresh blood sample taken from the pregnant woman within 24 hours, more preferably within 6 hours before use.

[0018] The blood cell separating system of the present invention comprises a primary separating device for mainly removing a large portion of the non-nucleated erythrocytes and maternal leukocytes which are a cellular fraction to be removed from the above-referenced blood sample. Specifically, the primary separating device should preferably be a density centrifugation device using a liquid having a specific density or a filter separation device.

[0019] The first embodiment of a primary separating device forming the blood cell separating system of the present invention is a density centrifugation device, wherein a density gradient fluid set to a predetermined density using an existing density adjusting agent such as sucrose and modified sucrose, glycerol or colloidal silica coated with polyvinylpyrrolidone is used. Examples include Ficoll-Paque, Ficoll-Hypaque and Percoll available from Pharmacia, or Histopaque available from Asuka-Sigma. The density gradient fluids generally used in the past for separation of blood components from the whole blood are set to a specific gravity of 1.077 (such as Ficoll-Paque and Histopaque-1077). In this case, centrifugation causes granulocytes and erythrocytes with a high specific gravity to go underneath the density gradient fluid, while monocytes and lymphocytes such as leukocytes rise above the density gradient fluid.

[0020] However, the present inventors discovered that when using the density gradient fluid with a specific gravity of 1.077, a portion of the fetal nucleated cells such as NRBC can spread into the density gradient fluid after centrifugation, so that much of the rare fetal nucleated cells are lost if only the layer containing the monocytes is taken, as is conventional. Thus, in the separating system of the present invention, a density gradient fluid having a specific gravity higher than 1.077 is preferably used as the primary separating device, and this can be used to prevent loss of fetal nucleated cells.

[0021] As shown by the below-described examples, increasing the specific gravity of the density gradient fluid will increase the recovery rate of fetal nucleated cells, but will simultaneously result in an increase in the intermixture of unwanted leukocytes. However, since the present invention can have a panning device as well as a secondary separating device which makes use of carbohydrate-lectin interactions as shall be described below, the intermixed leukocytes can be separated and removed to recover more fetal nucleated cells. Therefore, the density centrifugation device of the present invention comprises a centrifugal tube, a density gradient fluid with a specific gravity of 1.077-1.105, preferably 1.080-1.095 and more preferably 1.090-1.095 g/cm³ which is placed in the centrifugation tube, and a centrifuge separator. A blood sample taken from a pregnant woman is put into a centrifugation tube in which the density gradient fluid has been placed, and this is placed in the centrifuge separator to undergo centrifugation for 20-40 minutes at 500-2000 rpm (50-800 G), whereby a primary separated sample is obtained as a fraction of a layer placed above the density gradient fluid which contains large amounts of fetal nucleated cells.

[0022] In some cases, the primary separating device of the present invention may also have a panning device for further removing the intermixed leukocytes and the like. This panning device comprises, for example, a plate or the like such as a polystyrene dish which has been surface-treated with fetal calf serum (FCS), for separating and removing leukocyte components such as monocytes and granulocytes by making use of non-specific adhesion with respect to the plate.

[0023] The specific panning process may be such as has been conventionally used in this field. For example, the sample can be provided on the dish and incubated for a predetermined period, then the cells which have not adhered to the plate can be recovered as a suspension.

[0024] The dish should preferably be of a disposal plastic type, of which any type including commercially available plastic plate products such as the polystyrene dishes available from Nunc, Falcon, Iwaki Seiyaku or Sumitomo Bakelite may be used. FCS is added to these plates, then let stand, for example, for 2 hours at 4 °C, to coat the surface with proteins in the FCS. As a result, the hydrophobic plastic surface becomes hydrophilic, but any conditions can be used as long as a temperature of more than 37 °C at which the protein components in the FCS begin to change, or a

temperature which is below freezing is not used. Aside therefrom, it is also effective to use carbohydrates as the coating agents, of which PV-sugars (product of Netech, sold by Seikagaku Kogyo) which are easily coated onto plastic surfaces are suitable for use. The coating is obtained by dissolving PV-sugar powder into distilled water to obtain a 10-1000 µg/ml solution, of which those of 50-500 µg/ml are preferably used. This PV-sugar solution is put into the dish and left at room temperature for at least 30 minutes, whereupon the PV-sugar is adsorbed and the dish surface is hydrophilized.

5 Examples of carbohydrates which can form a PV-sugar include, as glucose types, PV-G (glucose), PV-MA (maltose), PV-GA (gluconic acid), PV-CA (cellobiose) and PV-Lam (laminaribiose); as galactose types, PV-LA (lactose), PV-LA-COOH (carboxylated lactose) and PV-MeA (melibiose); as well as PV-Man (mannobiose) and PV-GlcNAc (N-acetyl-chitobiose), but they may be of any type including naturally occurring polysaccharides, as long as they are carbohydrates which are capable of efficiently coating the dish surface. These are preferably used because they can easily coat polystyrene dishes, and do not lose or damage many fetal cells. In particular, those in which the carbohydrates forming the glycoconjugate polymer material are gluconic acid (PV-GA), melibiose (PV-MeA) or mannose (PV-Man) are particularly advantageous for their ability to achieve a high leukocyte adsorption and removal process in which the loss of fetal cells is held low. As coating agents, PV-sugars are preferable also for being synthetic polymers having a separating performance which is equivalent or superior to that of FCS, and being easier to obtain in stable lots and more easily stored than FCS.

10 [0024] After centrifugation, the primary separated sample is placed in the hydrophilized dish, and by letting stand for at least 5 minutes, the leukocyte components can be made to adhere at a higher rate to the dish surface, but they should preferably be left for 15 minutes to 2 hours in order to achieve an appropriate rate of leukocyte adherence and removal. During this period, any temperature at which the cells contained in the primary separated sample do not die

20 may be used, but a temperature of 18-37 °C at which the leukocytes can be actively led to adhesion is preferably used.

[0025] The panning process is not restricted to use of a plastic plate, and a glass plate or the like can be used by coating with FCS or PV-sugars as described above.

25 [0026] In general, contamination of a blood sample with many platelets, which can bind to lectins used in a secondary separation, after centrifugation may occur, which would adversely affect the preciseness of erythroblast analysis. However, panning treatment can effectively remove by adhering the platelets contaminated during blood sample preparation. Therefore, panning is a process effective for pre-removing excess granulocytes, monocytes and platelets, and offers highly precise erythrocyte separation.

30 [0027] Due to these panning treatments, even platelets, which have an extremely small specific gravity and grain size but an extremely high adhesion, are automatically removed from the primary separated sample obtained by the above-described centrifugation device so as to decrease to less than the detectable limit, thus making the sample rich in fetal nucleated cells with only a few non-nucleated erythrocytes and leukocytes such as lymphocytes remaining, and this can be made into the primary separated sample for the separating system of the present invention.

35 [0028] A second embodiment of a primary separating device forming the blood cell separating system of the present invention is a filter separating device. This filter separating device has a porous filter having a predetermined pore diameter generally used for separation of blood components, such that by passing a blood sample through this filter, the non-nucleated erythrocyte components which are deformable are normally passed through and removed, whereas the components including leukocyte components and erythroblasts, which are fetal nucleated cells, remain inside or on top of the filter material. By recovering the components remaining in the filter by means of a detergent fluid or the like, a primary separated sample containing fetal nucleated cells and leukocytes can be obtained.

40 [0029] The filter used herein is not especially restricted as long as it is capable of passing non-nucleated erythrocyte components (average size 7-8.5 µm), but physically or chemically capturing to block passage of leukocytes (average size 7-30 µm) and fetal nucleated cells (average grain size 8-19 µm), such that anything can be used, for example, non-woven fabrics which serve to capture by the form of aggregation or size of the fibers forming the filter, porous films with pore diameters controlled by elongating polymers, beads or spongy materials, as long as they have pore sizes within a range such as to capture orthochromatic erythroblasts having a minimum size of about 8 µm which are the most likely in the group of nucleated erythroblasts to be the object of recovery, yet allow non-nucleated erythrocytes to pass through by deformation. The material of the filter is not especially restricted, and may be of a synthetic or natural polymer such as fluoropolymers, polysulfones, polyesters, polyvinylacetals, polyvinylalcohols, polyamides, polyimides, polyurethanes, polyacrylics, polystyrenes, polyketones, silicones, polylactates, celluloses, chitosans, celluloses, silk or hemp, or inorganic materials including hydroxyapatite, glass, alumina, titania or metals such as stainless steel or titanium aluminum, which retain a certain level of adhesion with respect to leukocytes for maintaining the ability to collect leukocytes. Polyamides such as nylon, polyester, polyurethane, polyethylene, polypropylene, acrylic, polystyrene, polycarbonate, cellulose and hydroxyapatite are preferable in view of cost and production. The pore size of the filter should be 1.0-40 µm, preferably 2.0-20 µm and more preferably 3.0-10 µm, and if the filter is composed of fibers, the fiber diameter should be 1.0-30 µm, preferably 1.0-20 µm, and more preferably 1.5-10 µm.

45 [0030] Additionally, the above-mentioned materials forming the filter can be modified on the surface, and a type which does not inhibit the passage of non-nucleated erythrocytes and improves the retention of leukocytes is preferably used.

The fetal nucleated cells and leukocytes retained in the filter can be recovered by cleansing the filter. For example, since immature nucleated erythroblasts have a greater tendency to detach than do the leukocyte components, the targeted fetal nucleated cells can be selectively recovered by passing a detergent fluid through the filter in the direction opposite that used when filtering the blood sample. As the detergent fluid used here, any biological buffer solution or biological saline solution such as a transfusion or a culture solution can be used as long as it is capable of detach the fetal nucleated cells.

[0031] Accordingly, the filter separating device in the separating system of the present invention may, in addition to the above-described filter and detergent fluid, comprise a fluid feeding device such as a syringe or pump for supplying a detergent fluid to the filter or a fluid reservoir for collecting non-nucleated erythrocyte components flushed from the filter. The sample containing fetal non-nucleated cells obtained in this way is made into the primary separated sample according to the present invention.

[0032] The primary separated sample which has been separated by filter can then be made to undergo the above-described panning process to further reduce the number of leukocytes, and this can also be used as the primary separated sample.

[0033] In the blood cell separating system of the present invention, a secondary separating device is offered for removing residual non-nucleated erythrocytes and leukocytes from primary separated materials obtained in the manner described above to obtain a secondary separated sample with concentrated fetal nucleated cells. In this secondary separating device, the primary separated .. samples are incubated on a substrate with glycoconjugate polymers affixed to the surface, together with a predetermined concentration of lectins under conditions in which the cells are inactivated, thereby performing a method for concentrating and attaching fetal nucleated cells contained in the primary separated samples onto the substrate by means of selective binding with the lectins due to lectin-carbohydrate interactions (hereafter referred to as the "carbohydrate-lectin method").

[0034] The details of the carbohydrate-lectin method are described in WO00/58443. As the glycoconjugate polymers used here, those which incorporate a carbohydrate chain structure on a hydrophobic polymer main chain such as polystyrene is used. For example, PVLA, PVMA, PVMan, PVMcA, PVLACOOH, PVG, PVGlcNac and PVLam as described in WO00/58443 are especially preferable. Of these, it is preferable to select those having a carbohydrate chain structure recognized by the lectins being used.

[0035] On a substrate which is surface-modified by these glycoconjugate polymers, the primary separated samples and lectins are incubated under conditions which inactivate the cells to form fetal nucleated cell-lectin complexes, which are concentrated and attached to the substrate by means of carbohydrate-lectin interaction.

[0036] The lectins used are preferably those which recognize the carbohydrate chains expressed by the fetal nucleated cells, examples including galactose-recognizing lectins such as SBA, PNA, ECL, AlloA and VAA, glucose-recognizing lectins such as Con A, LcH and PSA, and mannose-recognizing lectins such as LCA, GNA and CPA, but are not limited thereto. Here, by adjusting the amount of lectins added, the fetal nucleated cells can be made to attach at a higher rate, so as to allow selective precision separation in which intermixed leukocytes from the mother are not attached. Specifically, when using a plastic substrate, the amount of lectins added with respect to a primary separated sample containing about 2×10^6 cells is preferably 8-35 µg, preferably 8-32 µg and more preferably 10-30 µg. Additionally, when using a glass substrate, the amount of lectins added with respect to a primary separated sample containing about 2×10^6 cells is preferably 10-200 µg, preferably 20-100 µg and more preferably 30-75 µg. Thus, while the optimum range of lectin concentrations used will change somewhat depending on the material of the substrate and type of lectin employed, the optimum concentration could be selected by one skilled in the art without undue experimentation.

[0037] When immobilizing blood cells from a maternal blood sample onto a substrate coated with glycoconjugate polymer (PV-LA), in the case using 300 µg/ml of lectin; many leucocytes and the like (strongly stained cells) were contaminated as shown in the microscopic photograph of Figure 2, whilst in the case using 8 µg/ml of lectin, few unnecessary cells such as leucocytes existed and the erythrocytes were selectively adhered as shown in microscopic photographs of Figures 3 and 4.

[0038] The incubation of the lectins and cells is performed under conditions in which the cells are inactivated, and such conditions enable the above-described selectivity to be. "Conditions in which the cells are inactivated" refer to conditions in which the fluidity and self-adhesiveness of the cell membranes are lowered, and typically are such that the temperature is adjusted to at least 0 °C to 37 °C, preferably 0-36 °C, more preferably 4-30 °C and most preferably 4-22 °C. However, these conditions are not limited to the above-mentioned low-temperature adjustments, and for example can be achieved by adding a reagent which suspends cell respiration, such as sodium azide at 37 °C.

[0039] Additionally, the incubation time is not particularly specified, so long as it is sufficient for the cells and lectins to form cell-lectin complexes, but is typically 0-120 minutes, preferably 0-90 minutes, more preferably 0-60 minutes. Here, "0 minutes" indicates that the subsequent step is begun immediately after mixing the primary separated sample and the lectins.

[0040] In this secondary separation, the cells adhering to the substrate after incubation (fetal nucleated cells possibly

including some non-nucleated erythrocytes) are separated by disposing of the unattached cells (leukocytes and the like) in the form of a cell suspension.

[0041] Accordingly, the secondary separating device according to the present invention comprises a substrate surface-modified with glycoconjugate polymers, lectins, and tools for inactivating the cells such as a cooling device or a reagent containing sodium azide, resulting in a secondary separated sample wherein fetal nucleated cells are attached to the substrate at a high density, and the unwanted components such as leukocytes are effectively removed.

[0042] In the blood cell separating system according to the present invention, the secondary separated sample, after being prepared by a preparing device to be explained, is finally made ready for genetic/chromosomal testing using the FISH method or the like. Therefore, by using the substrate in the second separating device as the slide plate in the FISH method or the like, a single substrate can be used for all processes from secondary separation to testing, which is very practical. Thus, if the slide plate used here is to be retained for microscopic examination, a flask, dish, cuvette or film having sufficient transparency so as not to inhibit the view in the microscope may be used, but when considering the general compatibility with microscopes, it is particularly preferable to use a chamber slide, to which a cover shell is attached during the secondary separation by lectins, the cover shell being removed after the secondary separation, and the attached fetal nucleated cells being dried and affixed, which can then be readily used directly in a FISH method.

[0043] The slide portion of the chamber slide may be made of any material which can be coated with the glycoconjugate polymers used in the above-mentioned carbohydrate-lectin process and viewed through a microscope, including organic materials such as polystyrene, polycarbonate, polysulfones, polyurethane and vinyl copolymers and the like, as well as inorganic materials such as glasses including silica. Additionally, when used for a FISH method, an organic solvent process at a high temperature must be used to denude the nuclei of the cells attached to the slide, so that a glass material which is not susceptible to deformation is preferably used.

[0044] Figure 5 indicates an example of substrate usable in the secondary separation and subsequent processes in the blood cell separating system of the present invention. The substrate described in Figure 5 contains a slide portion (1) constituting the bottom of the chamber and side walls (2) placed perpendicular to the slide portion (1). A layer (10) of glycoconjugate polymer is formed on the upper surface of the bottom of the chamber. The opening of the chamber can be sealed by a cap (3).

[0045] The blood cell separating system of the present invention comprises a preparing device for centrifugating the secondary separated sample under predetermined conditions to make a preparation.

[0046] Generally, when preparing a cell preparation on a glass slide for chromosomal examination or the like, a cell suspension fluid is pipetted, dropped onto a slide and air-dried, or a sample containing cells is spread over the slide, which is then dried to form a smear sample (for example, see JP-A H7-27682). However, the present inventors discovered for the first time that in order to attach cells from the secondary separated sample to a slide in a form suitable for examination, special centrifugation conditions must be employed.

[0047] Thus, the preparing device of the present invention comprises a centrifuge apparatus for centrifugation of the secondary separated sample, which can centrifugate the secondary separated sample under predetermined conditions while still remaining on the substrate. The conditions differ according to the substrate (slide) which is used.

[0048] If the substrate is a plastic chamber slide, the cover shell is removed after the cell suspension is removed, but in order to prevent the slide surface from drying, it should preferably be centrifugated after cleansing with a biological buffer solution or FCS containing albumin abundant in proteins at close to the biological concentration. Furthermore, FCS diluted with distilled water by preferably 1/2, more preferably 3/5 can be used to spread attached cells widely over the plate, thus enabling them to be readily viewed through a microscope, and are therefore particularly preferable for use. The range of dilution may be of any range as long as it is such as not to decompose the cells.

[0049] As for the centrifugal force, 100-1500 G is preferable for attaching cells to the plate, and 400-700 G is more preferable for being effective in shortening the centrifugation time. While the centrifugation time will change according to the centrifugal force, it should generally be about 1-15 minutes. If directly air-dried without centrifugation, the cells can often atrophy so as to preclude the obtainment of a good cellular image.

[0050] On the other hand, if a glass chamber slide is used, it is preferable in the above-described centrifugation process to use a two-step procedure of running a first centrifugation with a low centrifugal force, and a second centrifugation at a higher centrifugal force. Additionally, it is preferable to replace the cell suspension containing the unattached cells with FCS of preferably 1/2, or more preferably 3/5 dilution and perform the first centrifugation prior to removing the cover shell. This first centrifugation is preferably a light centrifugation at 10-300 G, preferably 10-150 G, and more preferably 10-50 G, whereby it is possible to further prevent the attached cells from detaching or from deforming when pressed. Next, the cover shell is removed and the second centrifugation is performed, thereby obtaining an affixed cellular image appropriate for testing. The second centrifugation should be at a centrifugal force of 25-300 G, preferably 30-200 G, and more preferably 35-130 G, whereby the reproducibility of an affixed cellular image without deformation is further increased.

[0051] As described above, by performing a centrifugation process in accordance with specific conditions which differ according to the material of the slide substrate, it is possible to obtain a test preparation composed of a slide substrate

on which fetal nucleated cells are affixed in a favorable state.

[0052] The blood cell separating system of the present invention further comprises an examination device for using test preparation in which fetal nucleated cells are concentrated and affixed to a substrate (slide) as described above for testing of the chromosomes and/or genes of the attached fetal nucleated cells.

5 [0053] The examination device may, for example, be a device for directly fluorescent labeling the chromosomes in the nuclei by means of the FISH method and viewing through a microscope, which is capable of detecting chromosome abnormalities such as aneuploidy, isochromosomes, translocation, deletion and reciprocal translocation according to the type of probe selected. Alternatively, the device may use the PCR method wherein the attached cells are recovered by peeling them by micromanipulation or laser dissection under a microscope, and amplifying the genes. The amplified 10 genes can further be screened on a gene chip, and the system of the present invention can be adapted to various types of chromosomal/genetic diagnosis.

15 [0054] As described above, by using the blood cell separating system of the present invention, rare fetal nucleated cells in the maternal blood, which are difficult to separate and concentrate using specific gravity or surface marker identification by antibodies, are separated and concentrated at a high precision and high density by combining primary separation by density centrifugation or filter separation under specific conditions with secondary separation based on carbohydrate chain recognition by lectins under specific conditions. In particular, according to the present invention, these rare fetal nucleated cells are directly attached, separated and concentrated on a substrate which can then be used for testing of nucleic chromosomes and genes, with the cells attached to the substrate maintaining a good cell form adaptable to the genetic/chromosomal examination. Thus, the present invention offers a method of producing a 20 test preparation of fetal nucleated cells capable of being directly used for testing in various chromosomal and/or genetic diagnoses by using the above-described system, as well as test preparations produced by the above-described method.

25 [0055] The method utilizing the system of the present invention is characterized by comprising a primary separation step of removing large amounts of unwanted cell components from rare cells contained in a sample, and a secondary separation step of separating and concentrating the rare cells with the object of further removing unwanted cell components from the primary separated samples, and for example, even if the secondary separation by the above-mentioned carbohydrate-lectin method is replaced by a separation method using antibodies specific to the rare cells, such a separation method and system would remain within the range of the present invention. Furthermore, aside from being fetal nucleated cells (fetal nucleated erythroblasts) intermixed in the maternal blood capable of diagnosing the state of and abnormalities in chromosomes and genes by testing after separation and concentration, the rare cells which 30 are targeted may be leukemia cells remaining after remission, or immature cells in the umbilical blood, and may be any type of cell capable of being concentrated on the slide substrate by selection by lectins or antibodies as used in the crude cell separation system or precision separation system offered by the present invention. The term "fetal nucleated cell" as used in the present invention shall be interpreted to encompass all rare cells which are targeted in this way.

35 Examples

40 [0056] Herebelow, the blood cell separation system of the present invention shall be described in detail with a focus on the setting of conditions for primary separation, secondary separation using the carbohydrate-lectin method, and preparing to maintain a good cell form.

Example 1: Primary Separation by Density Centrifugation

45 [0057] Histopaque (Sigma) was obtained to use as a density centrifugation reagent, to which sodium diatrizoate was added and 6 types of density gradient fluid with specific gravities adjusted to 1.077-1.105 were prepared. 7 cc of venous blood were taken from women who were 10-20 weeks pregnant, then centrifuged for 30 minutes in each density gradient fluid (20 °C, 1500 rpm). The cells collected around the boundary between the density gradient fluid and plasma component (upper layer) were recovered, then centrifugally rinsed with a biological buffer solution to obtain a crude separated sample with most of the non-nucleated erythrocytes and platelets removed.

50 [0058] The samples primarily purified under the various density conditions were secondarily separated by the carbohydrate-lectin method. As the substrate, a plastic chamber slide (2 wells, product of Nalgenunc) was used. The glycoconjugate polymer coated onto the substrate was PVMeA, with 12 µg of lectins (SBA) added for about 2×10^6 cells, then incubated for 30 minutes at 18 °C. The unattached cells were discarded in the form of a suspension, while the cells attached to the chamber slide were dried and stained with a Pappenheim stain. The stained cells were observed 55 through a microscope, and the orthochromatic erythroblasts (fetal nucleated cells) which had been separated and attached to the slide were counted. The results are shown in Table 1.

Table 1

| | Number of erythroblasts detected after secondary separation using carbohydrate-lectin method on sample primarily separated by centrifugation with various fluids (average of 10 samples) | | | | | |
|----|--|-------|-------|-------|-------|-------|
| | Density of Density Gradient Fluid | | | | | |
| | 1.077 | 1.080 | 1.090 | 1.095 | 1.100 | 1.105 |
| 5 | | | | | | |
| 10 | Number of orthochromatic erythroblasts detected on slide | 10.7 | 13.1 | 14.3 | 15.7 | 16.0 |
| 15 | Erythroblast detection ratio as compared with value for specific gravity 1.077 | 1.0 | 1.2 | 1.3 | 1.5 | 1.5 |
| 20 | | | | | | 2.1 |

[0059] As shown in Table 1, increasing the density of the density gradient fluid (Histopaque) clearly increased the number of erythroblasts detected after carbohydrate-lectin precision separation. This made it clear that the conventional separation of blood cells by density centrifugation using a density gradient fluid having a specific gravity of 1.077 lost the erythrocytes with a high specific gravity. On the other hand, while the number of orthochromatic erythroblasts increases when the specific gravity exceeds 1.095, a considerable increase is also observed in the number of intermixed leukocytes, as a result of which there were cases in which the detection of erythroblasts by microscopy was inhibited by a reduction in the precision separation efficiency due to the carbohydrate-lectin method.

Example 2: Additional Separation by Panning

[0060] A plastic chamber slide (4 wells, product of Nalgenunc) was treated with FCS or a 0.01 wt% aqueous solution of a glycoconjugate polymer (PV-Sugar) (product of Netech). As the glycoconjugate polymer, those having the structures of glucose, maltose, gluconic acid, N-acetylglucosamin, mannose, lactose or melibiose were used.

[0061] Density gradient centrifugation was performed on umbilical blood recovered after birth according to a standard method using Histopaque (d. 1.095), and the cells aggregating near the boundary between the Histopaque and plasma were collected. The samples were resuspended in RPMI1640 to which 10 wt% FCS was added, and inoculated onto the above-described wells whose surfaces were coated with FCS or glycoconjugate polymers. After incubation for 30 minutes at 37 °C, the unattached cells were recovered in the form of a cell suspension fluid, and the cells attached to the wells were stained with a Pappenheim stain to identify their types. The results are shown in Table 2. Table 2 shows the ratio between the erythrocyte fraction (including erythroblasts) and leukocyte fraction attached to the well surfaces with the respective types of coating, as well as the rate of adhesion of all inoculated cells.

Table 2

| | Adhesion of Blood Cells to Respective Wells (average of 5 samples) (%) | | | | | | | |
|----|--|---------|---------|---------|---------------|------------|---------|-----------|
| | FCS | Mannose | Glucose | Maltose | Gluconic Acid | Glucosamin | Lactose | Melibiose |
| 45 | 99.2 | 99.1 | 98.9 | 99.5 | 99.1 | 97.4 | 98.9 | 98.9 |
| 50 | Overall Adhesion Rate | 69.3 | 71.0 | 62.3 | 64.6 | 78.0 | 72.6 | 65.8 |

[0062] As shown in Table 2, it is clear that when panning with wells having their plastic surfaces coated with blood serum proteins (FCS) or glycoconjugate polymers, the cells of almost all blood corpuscles which attach are leukocytes. The attachment of the erythrocyte fraction is inhibited in wells treated with maltose, gluconic acid, FCS and mannose, while gluconic acid, glucosamin and mannose excel in the overall rate of adhesion which indicates the removability of

leukocytes. In this case, no erythroblasts were included in the attached erythrocyte fraction with the exception of a portion of he glucose type materials in which about 1 or 2 attached erythroblasts were observed.

[0063] Next, FCS was selected as a treatment agent for removing a suitable amount of leukocytes without much loss of the erythrocyte fraction, and the treatment effects of maternal blood were studied. A primarily separated sample obtained by density centrifugation using a density gradient fluid with a specific gravity of 1.095 was divided into two portions, one of which was panned under the above-described conditions and the other of which was not treated, then made to undergo a carbohydrate-lectin secondary separation under the same conditions as Example 1.

Table 3

| | |
|--|--|
| | Effects of panning on lectin secondary separation (average of 20 samples) (ratio compared to case of no panning) |
| Erythrocyte/Leukocyte Ratio per Field of Slide | 2.6 times |
| Number of Erythrocytes Detected on Slide | 2.6 times |

[0064] The erythrocyte/leukocyte ratio per field of the slide in Table 3 indicates the rate of removal of leukocytes in secondary separation, and the number of erythrocytes detected on the slide indicates the erythroblast recovery efficiency. Specifically, the results of a count of attached cells under the microscope are shown as a comparative ratio (multiple) of the cases where there is no panning and the case where panning has been performed. From the results in Table 3, it is dear that panning improves the selective separation and concentration efficiency of erythroblasts in the carbohydrate-lectin method. This is believed to be caused by a synergistic effect due to the fact that the panning has removed excess leukocytes, thus enabling the cells (erythroblasts) to be attached by the lectins to efficiently interact with the lectins so as to reduce adhesion misses, and that miscounting of erythroblasts has been reduced due to a decrease in the number of nucleated cells other than the erythroblasts during microscope observation.

[0065] The results from Table 3 show that even when compared with the case of a specific gravity of 1.095 in Table 1, the intermixture of leukocytes which block microscope observation is inhibited by panning, while making it possible to detect more erythroblasts than in the case of a specific gravity of 1.105 (no panning). Additionally, in the maternal blood, there was almost no loss of erythroblasts due to panning.

Example 3: Primary Separation by Filter Separation

[0066] Instead of the density centrifugation method of Example 1, a primary separation was performed using a filter comprising an unwoven polyester fabric with an average pore size of 8 µm. A sample of maternal blood was diluted with a biological buffer solution containing 1 wt% BSA, then passed through the filter by natural dripping. Next, the buffer solution alone was passed through a filter to rinse away the residual erythrocytes in the filter. Subsequently, the buffer solution was passed in the opposite direction with a syringe pump, and the unattached cells which did not pass through the filter were recovered. The cell fraction which did not pass through the filter but did not strongly adhere to the filter was taken as the primary separated sample, which was secondarily separated by the carbohydrate-lectin method. Table 4 shows the results with a primary separated sample obtained from the above-described filter, in the form of a comparative ratio (multiple) with respect to the results for the case where the cell fraction recovered by FCS panning is secondarily separated by the carbohydrate-lectin method.

Table 4

| | |
|--|--|
| | Effects of filtering on lectin secondary separation (average of 20 samples) (ratio compared to case of density centrifugation + panning) |
| Erythrocyte/Leukocyte Ratio per Field of Slide | 1.5 times |
| Number of Erythrocytes Detected on Slide | 2.4 times |

[0067] According to Table 4, both the erythrocyte/leukocyte ratio per field of the slide (leukocyte removal rate in secondary separation) and the number of erythroblasts detected on the slide (erythroblast recovery rate in secondary separation) are improved, thus dearly indicating that the selective separation and concentration efficiency of erythroblasts with the carbohydrate-lectin method can be improved by the filter process. The primary separation by the filter is believed to be due to non-nucleated erythrocytes which are capable of deforming being passed through the filter, and cells which are neither passed nor trapped by the filter being recovered by rinsing the filter. Thus, it was demonstrated that the loss of erythroblasts is further suppressed by using a primary separating method rather than the density cen-

trifugation method as the filtering method. While the erythrocyte/leukocyte selectivity was held to just a slight improvement, this is due to the fact that non-nucleated erythrocytes remaining in the filter were recovered by rinsing.

Example 4: Centrifugation in Preparing Step

[0068] A glass chamber slide (product of Nalgenunc) which is suitable for the FISH method was used to perform a secondary separation with the carbohydrate-lectin method. As for the primary separating conditions, the following were employed (same as Example 2).

Density Centrifugation: d, 1.095

FCS Panning

[0069] Comparisons were made between cases in which secondary separation by the carbohydrate-lectin method was followed by cases in which the cell suspension containing unattached cells was discarded, the chamber replaced with FCS, the cover shell removed, and the slide centrifugated (Experimental Conditions 1 and 2), and cases in which the cell suspension containing unattached cells was discarded, the chamber replaced with FCS, a first centrifugation immediately performed, and a second centrifugation performed on the slide after removing the cover shell (Experimental Conditions 3-11).

[0070] As the FCS, the raw liquid (1/1) was diluted by 1/2 with distilled water before use, and after the second centrifugation, the slides were air-dried at standard temperature. The cells on the slide were stained with a Pappenheim stain, and their respective stain appearances were compared. The results are shown in Table 5.

Table 5

| Slide Centrifugation Conditions | | | | | | Stain Appearance |
|---------------------------------|--------------------|---------------|-------------------|---------------------|---------------|------------------|
| Conditions | First Cent. (G) | Time (min) | Replaced Fluid | Second Cent. (G) | Time (min) | |
| 1 | | | 1/2 FCS | 1500 | 3 | F |
| 2 | | | 1/2 FCS | 200 | 10 | F |
| 3 | 200 | 5 | 1/2 FCS | 200 | 10 | D |
| 4 | 45 | 5 | 1/2 FCS | 200 | 10 | D |
| 5 | 25 | 5 | 1/2 FCS | 200 | 10 | C |
| 6 | 25 | 5 | 1/2 FCS | 130 | 10 | B |
| 7 | 25 | 5 | 1/2 FCS | 130 | 10 | C |
| 8 | 25 | 5 | 1/2 FCS | 70 | 10 | A |
| 9 | 25 | 5 | 1/2 FCS | 70 | 10 | C |

| Evaluation | Stain Appearance | |
|------------|--|------------------------------|
| | Form of Cell | Cytoplasm, Nuclear Structure |
| F | shrunken to points | Discernible |
| D | shrunken | Discernible |
| C | erythrocytes shrunken, leukocytes deformed | limit of discernibility |
| B | slight deformation | Clear |
| A | good | clear |

[0071] Unlike normal smear samples, the cells on a slide which have been secondarily separated by the carbohydrate-lectin method need to have the cells attached by centrifugation pressed, but when a glass slide is used as the substrate, a first centrifugation must be made in FCS solution at a low speed. Even in the second centrifugation after removal of the cover shell, relatively low-speed conditions retained good stain appearances. Additionally, while FCS- or BSA-added buffer solutions with a high protein concentration are effective as replacement fluids, diluted FCS under biological conditions induced swelling of the attached cells, and retained better stain appearances: For example, cells indicated suitable morphologies and clear structures of cytoplasm and nuclei under conditions 8, 10 and 11. In particular, under condition 11, in which 3/5 diluted FCS was used, almost no variation effects from samples such as storage periods or individuals were observed. When using plastic slides, an adequately good stain appearance was obtained

under the above-given Condition 1.

Effects of the Invention

- 5 [0072] According to the blood separating system of the present invention, the nucleated erythrocytes which are fetal nucleated cells that are contained in the maternal blood in extremely small amounts are selectively separated, concentrated and attached to a substrate. Additionally, by appropriately selecting the substrate used, the processing from the secondary separation step to the preparing step can be performed on a single substrate to obtain a test preparation which is suitable for chromosomal/genetic diagnosis, and this test preparation can be directly applied to testing means
 10 such as the FISH method. Accordingly, a fetal nucleated cell test sample with a high clinical value for prenatal diagnosis can be produced conveniently and at a low cost without invading the maternal body.

Claims

- 15 1. A blood cell separating system comprising:
- 20 a primary separating device for removing mainly non-nucleated erythrocytes, leukocytes and platelets from a blood sample taken from a pregnant woman to obtain a primary separated sample;
- 25 a secondary separating device for removing residual non-nucleated erythrocytes and leukocytes from the primary separated sample obtained from said primary separating device, to obtain thereby a secondary separated sample with concentrated fetal nucleated cells, the secondary separating device involving incubation of said primary separated sample, under conditions which inactivate the cells, along with a predetermined concentration of lectins, on a substrate having glycoconjugate polymers affixed to the surface thereof, thereby selectively binding fetal nucleated cells contained in said primary separated sample with said lectins to thereby concentrate and attach them to said substrate by means of a lectin-carbohydrate interaction to form a secondary separated sample; and
- 30 preparing device for preparing the secondary separated sample obtained from said secondary separating device, the preparing device involving centrifugation, under predetermined conditions, of said substrate on which said fetal nucleated cells have been concentrated and attached, to thereby obtain a test preparation.
- 35 2. A blood cell separating system in accordance with claim 1, wherein said primary separating device is a density centrifugation device using a density gradient fluid having a density at least exceeding 1.077 mg/cm³.
- 40 3. A blood cell separating system in accordance with claim 2, further comprising panning device for removing leukocytes intermixed in the sample containing fetal nucleated cells obtained by said density centrifugation device.
- 45 4. A blood cell separating system in accordance with claim 1, wherein said primary separating device is a device for separating non-nucleated erythrocytes as well as leukocytes using a filter.
- 50 5. A blood cell separating system in accordance with claim 1, wherein the conditions which inactivate the cells in said secondary separating device are low-temperature conditions of at least 0 °C and less than 37 °C, or conditions which suspend cell respiration.
- 55 6. A blood cell separating system in accordance with claim 1, wherein the centrifugation conditions in said preparing device are centrifugation for 1-15 minutes at 100-1500 G when using a plastic substrate, and a first centrifugation of 1-10 minutes at 10-300 G followed by a second centrifugation of 5-15 minutes at 25-300 G when using a glass substrate.
- 50 7. A blood cell separation system in accordance with claim 1, wherein a substrate used in said preparing device is obtained by substituting the suspension contained in the secondary separated sample with FCS diluted to 1/2, preferably 3/5.
- 55 8. A blood cell separating system in accordance with claim 1, further comprising an examination device for testing the chromosomes and/or genes in fetal nucleated cells contained in a test preparation obtained from said preparing device.
9. A blood cell separating system in accordance with claim 8, wherein said examination device comprises a staining

tool for staining chromosomes in the nuclei of fetal nucleated cells contained in the preparation.

10. A blood cell separating system in accordance with claim 9, wherein the staining of the nuclei in said staining means
is due to the FISH method.

- 5 11. A blood cell separating system in accordance with claim 8, wherein said examination device includes a chromo-
some testing device using an optical microscopy or a fluorescent microscopy.

- 10 12. A blood cell separating system in accordance with claim 8, wherein said examination device comprises tools for
amplifying genes extracted from the chromosomes of fetal nucleated cells contained in the preparation.

- 15 13. A blood cell separating system in accordance with claim 12, wherein said amplifying tools involve materials for
performing the PCR method.

- 15 14. A blood cell separating system in accordance with claim 12, wherein said examination device contains a device
for screening amplified genes.

15. A method for producing a test preparation for prenatal fetal chromosomal and/or genetic diagnosis, comprising:

- 20 a primary separating step of removing mainly non-nucleated erythrocytes, leukocytes and platelets from a
blood sample taken from a pregnant woman to obtain a primary separated sample;
a secondary separating step of incubating said crude separated sample, under conditions which inactivate
the cells, along with a predetermined concentration of lectins, on a substrate having glycoconjugate polymers
affixed to the surface thereof, thereby selectively binding fetal nucleated cells contained in said primary sep-
arated sample with said lectins to thereby concentrate and attach them to said substrate by means of a lectin-
carbohydrate interaction, so as to selectively remove residual leukocytes in said primary separated sample to
obtain a secondary separated sample having the desired fetal nucleated cells in concentrated form; and
a preparing step of performing centrifugation, under predetermined conditions, of the secondary separated
sample in which said fetal nucleated cells have been concentrated and attached.

- 30 16. A method in accordance with claim 15, wherein said primary separating step is performed by density centrifugation
using a density gradient fluid having a density at least exceeding 1.077 mg/cm³.

- 35 17. A method in accordance with claim 16, further comprising a step of panning the sample containing fetal nucleated
cells obtained by said density centrifugation means to remove intermixed leukocytes.

18. A method in accordance with claim 15, wherein said primary separating step is performed by separating non-
nucleated erythrocytes as well as leukocytes using a filter.

- 40 19. A method in accordance with claim 15, wherein the conditions which inactivate the cells in said precision separating
step are low-temperature conditions of at least 0 °C and less than 37 °C, or conditions which suspend cell respi-
ration.

- 45 20. A method in accordance with claim 15, wherein the centrifugation conditions in said preparing step are centrifu-
gation for 1-15 minutes at 100-1500 G when using a plastic substrate, and a first centrifugation of 1-10 minutes
at 10-300 G followed by a second centrifugation of 5-15 minutes at 25-300 G when using a glass substrate.

- 50 21. A method in accordance with claim 15, wherein a substrate in which the suspension contained in the secondary
separated sample is substituted with FCS diluted to 1/2, preferably 3/5 is provided to said preparing step.

22. A test preparation for prenatal fetal chromosomal and/or genetic diagnosis produced by a method in accordance
with any one of claims 15-21.

- 55 23. A substrate for a blood cell separating system in accordance with any one of claims 1-14, having glycoconjugate
polymers affixed to the surface thereof.

24. A blood cell separating method comprising a primary separating step of removing most of the unneeded cellular
components included in a sample containing rare cells; and a secondary separating step of separating and con-

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centrating the targeted rare cells by further removing unneeded cell components from the primary separated sample.

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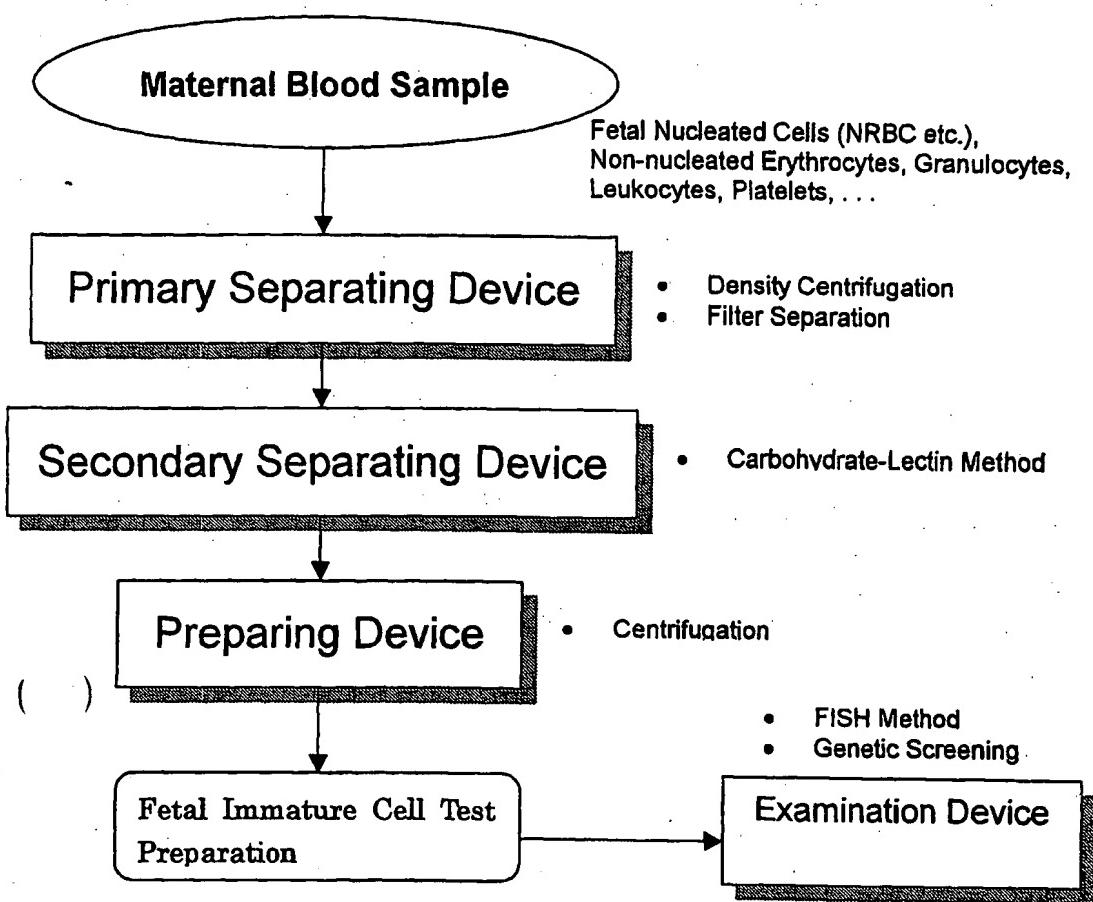


FIG. 1

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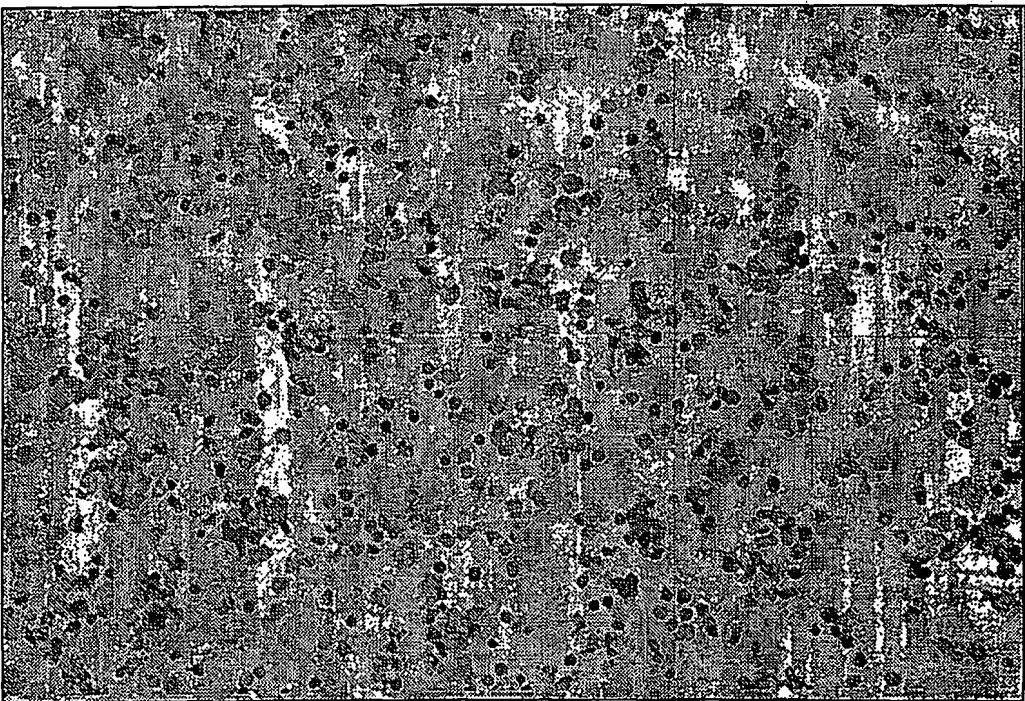


FIG. 2

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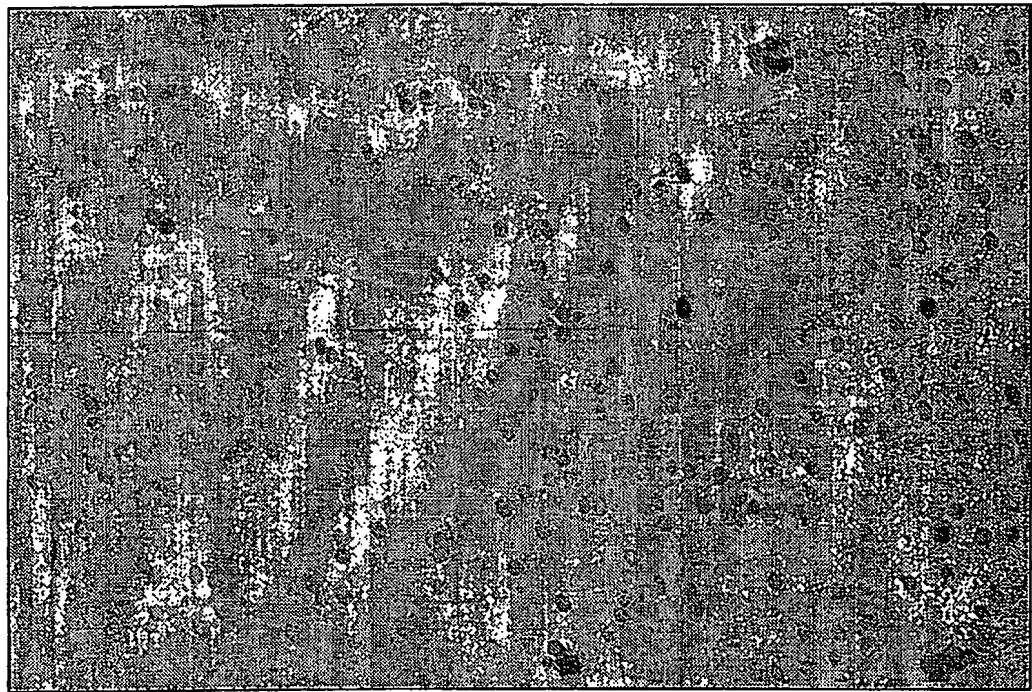


FIG. 3

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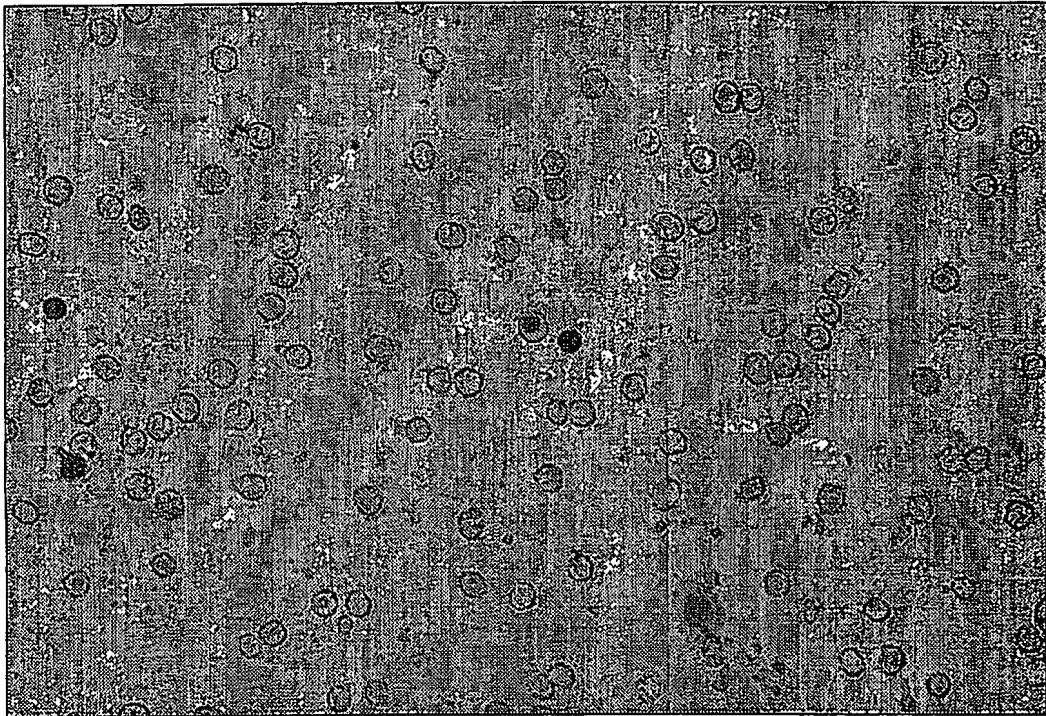


FIG. 4

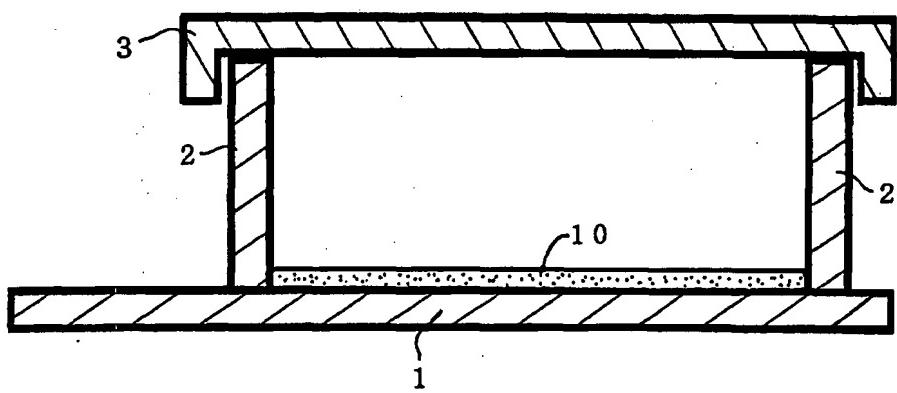


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP02/12839

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl⁷ G01N33/48, 33/49

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl⁷ G01N33/48, 33/49Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Toroku Jitsuyo Shinan Koho 1994-2003
Kokai Jitsuyo Shinan Koho 1971-2003 Jitsuyo Shinan Toroku Koho 1996-2003

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | WO 00/58443 A (Netech Inc.), 05 October, 2000 (05.10.00), & EP 1167518 A | 1-24 |
| Y | JP 2000-502892 A (Dendorecon Corp.), 14 March, 2000 (14.03.00), & EP 778944 A | 1-3, 5-17, 19-24 |
| Y | JP 10-508190 A (Activated Cell Therapy, Inc.), 18 August, 1998 (18.08.98), & EP 778944 A | 1-3, 5-17, 19-24 |
| Y | JP 10-508695 A (Phoenix Medical Ltd.), 25 August, 1998 (25.08.98), & EP 791175 A | 4, 18 |

 Further documents are listed in the continuation of Box C. See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family |
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| Date of the actual completion of the international search 10 March, 2003 (10.03.03) | Date of mailing of the international search report 25 March, 2003 (25.03.03) |
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| Name and mailing address of the ISA/ Japanese Patent Office | Authorized officer |
| Facsimile No. | Telephone No. |

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP02/12839

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|--|-----------------------|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | JP 8-319300 A (Kanagawa Academy of Science and Technology), 03 December, 1996 (03.12.96), (Family: none) | 1-24 |

Form PCT/ISA/210 (continuation of second sheet) (July 1998)



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(54) System for characterising a fluid, microfluidic device for characterising or analysing concentration components, a method of characterising or analysing such concentrations and a measurement device

(57) The present invention relates a system for characterising a fluid, comprising a microfluidic device and a measurement device, to the microfluidic device, to the measurement device, to the method of characterising or analysing a concentration of a component.

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Description

[0001] The present invention relates a system for characterising a fluid, comprising a microfluidic device and a measurement device, to the microfluidic device, to the measurement device, to the method of characterising or analysing a concentration of a component.

[0002] Analytic detection of particles, molecules and especially biomolecules, e.g., proteins, nucleic acids, hormones and the like, is fundamental to diagnostics as well as to molecular biology. In many applications, it is desirable to detect the presence of at least one particular molecule in a sample. Analytic detection is also used, e.g., in disease diagnosis and drug development, to determine the presence of a particular antibody or protein, e.g., in a blood sample or large chemical library. Detection of particles, molecules and biomolecules is therefore of fundamental value in, e.g., diagnostic medicine, archaeology, anthropology and criminal investigation. To meet these needs many techniques, e.g., DNA blotting, RNA blotting, protein blotting, and ELISA assays, have been developed to detect the presence of a particular molecule or fragment in the midst of a complex sample containing similar molecules.

[0003] More recently, new and faster microfluidic methods of performing biological assays in microfluidic systems have been developed, such as those described by the applications of Farce et al., "High Throughput Screening Assay Systems in Microscale Fluidic Devices" WO 98/00231 and in Knapp et al., "Closed Loop Biochemical Analyzers" (WO 98/45481; PCT/US98/06723). For example, high throughput methods for analyzing biological reagents, including proteins, are described in these applications.

[0004] Improved methods as well as the availability of fast, simple reliable and cheap detection systems for affinity assays are, accordingly, desirable, particularly those which take advantage of high-throughput, low cost microfluidic systems.

[0005] US-A-6,444,461 discloses integrated systems, apparatus, software, and methods are provided for performing biochemical analyses, including DNA sequencing, genomic screening, purification of nucleic acids and other biological components and drug screening. Microfluidic devices, systems and methods for using these devices and systems for performing a wide variety of fluid operations are provided. The devices and systems are used in performing fluid operations that require a large number of iterative, successive or parallel fluid manipulations, in a microscale, or sealed and readily automated format.

US-A-6,235,175 discloses microfluidic devices that incorporate improved recess and reservoir geometries, as well as methods of using these devices in the analysis, preparation, or other manipulation of fluid borne materials, to achieve higher throughputs of such materials through these devices, with lower cost, material and/or space requirements. It is mainly aimed at improved re-

cess and reservoir geometries. This is necessary as the dimensions are relatively small, that is in the order of 1-100 μ m.

US-A-6,479,299 discloses microfluidic devices having predisposed assay components for increased throughput and prolonged shelf life. The methods involve flowing a first component of a biochemical system in a first of the at least two intersecting recesses. At least a first test compound is flowed from a second recess into the first recess whereby the test compound contacts the first component of the biochemical system. An effect of the test compound on the biochemical system is then detected. It uses electrokinetic flow.

US-A-6,613,581 discloses methods of detecting a component of interest, such as a protein, in a microfluidic system. The methods include the use of a component-binding moiety specific to the component of interest, such as an antibody, to detect the component of interest. Also included are microfluidic devices and integrated systems for performing such assays, including devices utilizing flowable or fixed particle sets.

US-A-6,644,944 discloses microfluidic fluid control devices. One microfluidic fluid control device can be used as a uni-directional valve within a microfluidic system.

Said US-A-6,644,944 also teaches a microfluidic pump mechanism having two unidirectional valves separated by an expandable reservoir. Such devices may be formed in multiple layers and utilize flexible membranes.

US-A-6,408,878 discloses a method of fabricating an elastomeric structure, comprising: forming a first elastomeric layer on top of a first micromachined mold, the first micromachined mold having a first raised protrusion which forms a first recess extending along a bottom surface of the first elastomeric layer; forming a second elastomeric layer on top of a second micromachined mold, the second micromachined mold having a second raised protrusion which forms a second recess extending along a bottom surface of the second elastomeric layer; binding the bottom surface of the second elastomeric layer onto a top surface of the first elastomeric layer such that a control recess forms in the second recess between the first and second elastomeric layers; and positioning the first elastomeric layer on top of a planar body such that a flow recess forms in the first recess between the first elastomeric layer and the planar body.

US-A-6,086,740 discloses multiplexed microfluidic devices including a plurality of modular microfluidic elements, all of which are attached to a common frame or body, which itself includes one or more common input elements that are connected to corresponding input elements within several or each of the microfluidic modules for use in common control and/or common detection operations for each of the modules.

[0006] The state of the art is aimed at very small structures (containing typically less than 10 μ l fluid). Disadvantages of such small structures are that fluids tend to clog and/or that upon flow bubbles in the fluid may be

formed.

[0007] Furthermore the volumes of samples and /or the constituents therein are often too large to flow through the small (nanoscale) structures. Not only impurities present in the sample but also components, such as red blood cells, tend to clog the structures and/or hinder the flow severely.

[0008] Furthermore, it is clear that the fabricating techniques for very small structures are quite complicated.

[0009] Another disadvantage is that the body used does not comprise recesses on several of its surfaces, e.g. on both sides of a card-like body. This makes it difficult to separate or position functions, such as a pumping function from a chamber function.

[0010] A next disadvantage is that when electrokinetic flow is applied, it will typically function sub-optimally, that is the components do not flow according as intended. Amongst others electroosmotic flow interferes with the electrokinetic flow.

[0011] Another disadvantage is that the flow can not be controlled sufficiently, especially the amount of fluid to be flown as well as the velocity of the flow.

[0012] A next disadvantage is that the microfluidic devices of the state of the art are so small that they are difficult to handle.

[0013] A next disadvantage is that the characterisation of the components present in the microfluidic devices are difficult to be determined, as it is difficult to get access to components present and/or the measuring device to be used is not optimised for microfluidic devices.

[0014] Another disadvantage is that the microfluidic devices are relatively static systems. They allow for simple operations, that is typically reacting one fluid with another and typically only once.

[0015] Another disadvantage is that the microfluidic devices are in general not supplied with fluids, that may contain chemicals, and if so only a very limited set is present.

[0016] An additional disadvantage is that the microfluidic devices mentioned above are not dedicated to specific uses. For instance they do not contain a moiety to which a label or component present can be bound.

Detailed description of the invention

[0017] The present invention relates a system for characterising a fluid, comprising a microfluidic device and a measurement device, to the microfluidic device, to the measurement device, to the method of characterising or analysing a concentration of a component.

[0018] In a first embodiment the present invention relates to a system for characterising or analysing a fluid, which fluid is suspected to comprise at least one component to be characterised or analysed, comprising a microfluidic device, at least one pump for transporting the fluid and a measurement device which is arranged

to characterise or analyse the fluid in use present in the microfluidic device,

which microfluidic device comprises at least one body (11), wherein the body has at least one surface, wherein the at least one surface has at least a part of the recess for containing the fluid in the microfluidic device and/or transporting the fluid in the microfluidic device through at least a part of the microfluidic device,

wherein the body has at least one provision for an inlet (15) and at least one provision for an outlet (18),

wherein at least a part of said recess (16) is a reaction chamber, which reaction chamber comprises a moiety that binds to the at least one component that is suspected to be present and that is to be characterised or analysed, which reaction chamber is arranged for characterising or analysing the at least one component, wherein at least a part of said recess is a fluid connection (14) between the at least one provision for an inlet (15) and the at least one provision for an outlet,

wherein at least a part of said recess (17) is a pump chamber, wherein at least the reaction chamber, pump chamber and fluid connection are sealed from the environment by at least one cover layer (12, 13).

[0019] A general layout of the microfluidic device is given in figure 1. It contains several recesses (14-17) on a body (11). The recesses are formed on both sides of the body. The fluid to be characterised or measured is brought into the provision for an inlet (15). Then, it

flows to the reaction chamber (16). In the reaction chamber, a component that is suspected to be present can bind to a moiety, present in the reaction chamber (16). It further contains a pump chamber (17), which can be arranged to a pump (not visible). The pump forces the fluid to flow from the pump chamber to the reaction chamber or vice versa. The term "component" as used herein refers to a component that itself binds to a label or a moiety present in the microfluidic device, or to a chemical part of that component, of which at least one part binds to a label or a moiety present, or to a component which is labeled in the microfluidic device, which labeled component binds to the moiety present in the microfluidic device, or to a component that causes a detectable signal by itself, by a chemical reaction with another component, or by a component formed here out,

whereby the detectable signal may be chemiluminescent flash or flow, colorimetric, fluorescent and time-resolved fluorescent.

[0020] The term "fluid" as used herein refers to liquid compositions that flow at operating pressure and temperature.

[0021] The term "pump" as used herein refers to a combination of an actuator, a displacement volume and at least one means for transferring the variation in pressure of the actuator towards the displacement volume. The means for transferring the variation can be a membrane. The displacement volume is referred to as pump chamber. The pump further comprises means for con-

trolling the actuator. The pump chamber typically has a volume of 1 -1000 µl, preferably of 10-100 µl.

[0022] The term "inlet" as used herein refers to a provision through which a fluid or a gas may pass. The direction of the fluid or gas is intended to be from the environment to a recess. It typically has a volume of 1-1000 µl, preferably of 1-10 µl.

[0023] The term "outlet" as used herein refers to a provision through which a fluid or a gas may pass. The direction of the fluid or gas is intended to be from a recess to the environment. It typically has a volume of 1-1000 µl, preferably from 1-10 µl.

[0024] The term "fluid connection" as used herein is a recess sealed by a cover layer. It is to be interpreted in a broad sense. Thus, it is not intended to be restricted to elongated configurations where the transverse or longitudinal dimension greatly exceeds the diameter or cross-sectional dimension. Rather, recesses are meant to comprise cavities and/or tunnels of any desired shape or configuration through which fluids may be directed. A cavity may, for example, comprise a flow-through cell where fluid is to be continuously passed or, alternatively, a chamber for holding a specified, discrete amount of fluid for a specified amount of time. A "fluid connection" may be filled or may contain internal structures comprising fluid diodes, valves or equivalent components. Its volume is from 1 to 1000 µl, preferably from 10-100 µl.

[0025] The term "microfluidic" as used herein is to be understood, without any restriction thereto, to refer to structures or devices through which fluid(s) are capable of being passed or directed, wherein one or more of the dimensions is less than 500 microns.

[0026] The term "recess" as used herein refers to a "fluid connection" type structure that is present on a surface of the body of the microfluidic device. The body substantially surrounds it. A recess in use is at least partly sealed by at least one cover layer, except for inlet and outlet provisions. It may also refer to part of a recess, especially in the case of chambers, meandering fluid path and fluid connection.

[0027] The term "chamber" as used herein refers to part of a covered recess in the body, which has a volume of 1-1000 µl, preferably of 10-100 µl. It is capable of for instance comprising a fluid, a binding-moiety etc. From the above it is clear that a chamber is also used as a fluid connection.

[0028] The term "reaction chamber" as used herein refers to a chamber used for reacting components and/or for binding at least one component to a moiety that is present in the reaction chamber. Furthermore, the reaction chamber can be arranged with the measurement device. It has a volume of 1-1000 µl, preferably of 10-100 µl, more preferably of 10-30 µl.

[0029] The term "meandering fluid path" as used herein refers to part of a fluid connection. It is a channel type part of a recess, which channel forms several bends. Hereby a relatively long channel occupies only a limited amount of surface on the microfluidic device.

It has a volume of 1 -1000 µl, preferably of 10-500 µl, more preferably of 50-200 µl.

[0030] The term "body" as used herein refers to a solid material. The solid material has at least one surface and can be of any shape. A preferred shape has the dimensions of a "credit-card". At least one surface of the body comprises at least a part of a recess. In a preferred embodiment of the present invention the top and bottom side of the body comprise at least one recess. The at least one recess of the topside is in fluid connection with the at least one recess of the bottom side. The solid material should allow for manufacturing techniques to form recesses on the surface of the body. Such manufacturing techniques are for instance moulding, injecting moulding, hot embossing and lithographic processes, optionally combined with etching techniques. Further, the material preferably is stable and chemically resistant to the fluids used in the microfluidic device. It furthermore preferably has the desired physical properties, such as hydrophilicity and a smooth surface after manufacturing. The material used as body is typically a polymer or silicon or glass. A suitable polymer is selected from the group consisting of latex, rubber, polyesters, polycarbonates, polyalkanes, polyalkenes, polytetrafluoroethylenes, polypropylenes, polyimides, polymethylmethacrylates, silicones, polymethylmethacrylate (PMMA), PEEK, polystyrene, PDMS, and polyesters. A preferred material is polymethylmethacrylate (PMMA).

[0031] The term "cover layer" as used herein refers to a material that is used to seal recesses from the environment. The material used as cover layer is typically a polymer or silicon or glass. A suitable polymer is selected from the group consisting of latex, rubber, polyesters, polycarbonates, polyalkanes, polyalkenes, polytetrafluoroethylenes, polypropylenes, polyimides, polymethylmethacrylates, silicones, polymethylmethacrylate (PMMA), PEEK, polystyrene, PDMS, and polyesters. A preferred material is polymethylmethacrylate (PMMA).

[0032] Typically the moiety that binds a component is attached to the cover layer, covering the reaction chamber. This has the advantage that the moiety can be deposited on the cover layer, prior to covering the reaction chamber recess with this cover. In figure 2 this configuration is shown. The cover layer (12) has an adhesive (22) to which the moiety (23) is attached. The reaction chamber itself (16) is a recess in the body (11). Further, a second cover layer (13) is visible. In another embodiment the moiety can be present in the form of magnetic and/or non-magnetic antibody coated particles. The moiety that binds is chosen from the group consisting of a nuclear receptor, an intracellular receptor, a solubilized receptor, an antibody, an antigen, an enzyme, avidin, a polynucleotide and a polysaccharide.

[0033] In many of the above described embodiments it has been a goal to minimize the dimensions. The inventors of the present invention have found however

that such a minimization encompasses all sorts of problems, such as clogging, bubble-formation, manufacturing problems and so on.

[0034] The present invention has a first advantage that it can optimally use the surface of the body, e.g. both sides of a credit-card shaped body, to form recesses. This makes it possible to separate various functions of the system, such as the pump function and the receiving function. In a preferred embodiment of the invention the energy transfer (pump function) is located at one side of the body and the provision for an inlet and the reaction chamber are located on the other side. The pump function generally requires a relative large amount of space, even with the micro-sized pumps that now become available.

[0035] The present invention is not particularly aimed at reducing the size; it rather provides a microfluidic device that is easy to operate. For instance in a preferred embodiment of the invention the microfluidic device has size of that is similar to that of a credit card, e.g. 85 by 60 by 1 mm³.

[0036] The present invention further has the advantage that it provides a combination of a microfluidic device with a compatible measurement device. For instance the microfluidic device is filled with a fluid to be characterised or analysed. After the pump forces the fluid to flow from the pump chamber to the reaction chamber, the fluid can be characterised by the measuring device by arranging the microfluidic device with the measuring device.

[0037] In a preferred embodiment the system of the invention comprises a measurement device for characterising the fluid, wherein the measurement device is arranged to obtain information based on an optical technique selected from the group consisting of fluorescence, chemiluminescence, time resolved fluorescence, time resolved chemiluminescence, colorimetry or a combination thereof, or from the group consisting of magnetic measurements, resistivity measurements, capacity measurements, surface plasma resonance (SPR) measurements, or a combination thereof. This embodiment has the advantage that the measurement device can easily be arranged to the microfluidic device. A preferred embodiment radiates the reaction chamber and detects emitted radiation. An optical measurement device is arranged to obtain information based on a technique selected from the group consisting of fluorescence, chemiluminescence, time resolved fluorescence, or a combination thereof. A preferred embodiment uses a fluorescence technique.

[0038] In another embodiment the system of the invention characterises the concentration of at least one component present in the fluid. Preferably it is used to characterise one component, which has the advantage that the system can be fully optimised to characterise this one component. For instance the moiety present in the reaction chamber, the wash fluid, the detection are optimised. It provides microfluidic devices comprising all

necessary material in the device.

[0039] In a preferred embodiment the system of the invention comprises at least one pump. This pump can be present on the microfluidic device or in the measurement device. Preferably this pump is a piezo-pump. The present invention makes use of a piezo pump in a structure, which has as further advantage that it enables the fluid to be directed from a part of the recess to another recess. The present invention further uses the piezo pump in order to perform all kinds of pumping functions, for instances to move fluids in controlled amounts from a part of the recess to the other, to perform pumping cycles, to optimise piezo frequencies with respect to the dimensions of the recesses, to permit time intervals between pumping etc. The at least one piezo-pump preferably operates at a frequency up to 40 kHz. The frequency may also be used to reverse the preferred flow direction of the fluid diode. The man skilled in the art will appreciate the possibilities of such a pump and will apply the pump in such a way to fulfil the requirements of the specific application.

[0040] In another embodiment the system is disposable all together. In extreme situations, such as emergency or war, there may be a need to identify the status 25 of a patient on short notice, whereas the desire to maintain the measurement device is not an issue. Such a disposable system has in such a situation the advantage of providing a dedicated and quick answer to the status of a patient.

[0041] In a next embodiment of the invention a microfluidic device arranged for use in the system of the invention is used, which microfluidic device comprises at least one body (11), wherein the body has at least one surface, wherein the at least one surface has at least a 35 part of the recess for containing the fluid in the microfluidic device and/or transporting the fluid in the microfluidic device through at least a part of the microfluidic device,

wherein the body has at least one provision for an inlet 40 (15) and at least one provision for an outlet (18), wherein at least a part of said recess (16) is a reaction chamber, which reaction chamber comprises a moiety that binds to the at least one component that is suspected to be present and that is to be characterised or analysed, which reaction chamber is arranged for characterising or analysing the at least one component, wherein at least a part of said recess (16) is a fluid connection (14) between the at least one provision for an inlet (15) and the at least one provision for an outlet (18),

50 wherein at least a part of said recess (17) is a pump chamber, wherein at least the reaction chamber, pump chamber and fluid connection are sealed from the environment by at least one cover layer (12, 13).

[0042] In a preferred embodiment of the microfluidic device two cover layers form one part, which make it easier to seal the microfluidic device. This is an advantage in the manufacture of the microfluidic device.

[0043] In a next embodiment of the microfluidic device it further comprises a filter in at least one provision for an inlet. The filter is used to hold particles and/or components that adversely interfere in the characterisation or analyses of the fluid in the measurement device. The filter is for instance a particle filter, such as a Millipore™ filter with an intended hole-size, or a chemical compound that reacts or binds to undesired components, thereby immobilising these components.

[0044] In a next embodiment of the microfluidic device it further comprises at least a part of the recess for a washing fluid and at least a part of the recess for collecting waste fluid. This has the advantage that after moving the fluid from the provision for an inlet to the reaction chamber and after reaction in the reaction chamber, the reaction chamber is washed with a washing fluid. Hereby is the reaction chamber cleaned, which has the advantage that the subsequent measurement is not or less adversely effected by other components present in the fluid to be characterised.

[0045] In a next embodiment of the microfluidic device it further comprises at least a part of the recess which comprises at least one label fluid, which label binds to the moiety in the reaction chamber and/or to the at least one component to be characterised or analysed. In figure 3 such a layout is given. It shows a top view of the microfluidic device. It should be noted that the bottom side of the microfluidic device comprises also a number of recesses. The bottom side is brought into an arrangement with the pump-actuator. A first glance at the figure immediately indicates the intense use of the surface of the body. Not only do reaction chamber(s) occupy space, but also the meandering fluid path type recesses do. These meandering fluid path type structures have the advantage that they contain a relative large volume on the side, which is combined with the possibility to flow very well controlled amounts of fluid on the other side. In a preferred embodiment a meandering fluid path type structure contains from 1-1000 µl of fluid. A further advantage with respect to a chamber is that the meandering fluid path can be emptied almost completely, whereas a chamber always has some residual liquid. Further, the meandering fluid path has less leakage as compared to a chamber. The fluid to be characterised is transferred to the provision for an inlet (15). This provision for an inlet is in fluid connection with the central reaction chamber (16). It is optional to have other reaction chambers present (16), which serve similar functions as the first chamber. Not visible in the layout of figure 3 are various measures taken to improve the flow of the fluid to be characterised and analysed and the other fluids used. These measures are amongst others a special design of the provision for an inlet and of the fluid connection between the provision for an inlet and the reaction chamber, as well as measures taken to improve the fluid flow in reaction chamber. This has the advantage that the fluids in the reaction chamber mix better, thereby improving the reaction-rate and minimis-

ing the required amount of fluid to be obtained an intended result. The recesses that contain wash fluid, label fluid, sample fluid (fluid to be characterised or analysed) and waste fluid are associated with a pump, such

5 as a piezo-pump. The fluid flows through one of the fluid connections. The provision for an inlet (15) is used to insert the fluid to be characterised or analysed. Fluid connections (33-35) are used to transfer the label fluid. Fluid connections (36-38) are used to transfer the wash fluid. Fluid connection (39) is used to collect the waste fluid. This embodiment has the advantage that it comprises all necessary fluids in one single body. Furthermore the fluid connections and the arrangement of the pump enable complicated reaction sequences, involving one or more label steps and one or more washing steps.

[0046] In figure 4 another layout is given. The dimension of this layout is 60 mm by 49 mm. The recess on the bottom surface as well as the contact area of the 20 pump actuator is projected onto the front side. Figure 4 shows a provision for an inlet (15), wherein the fluid to be characterised or analysed is injected. The fluid is moved from the inlet (15) towards the reaction chamber (16) by means of a pump (17), located on the bottom side, in fluid connection with the front side. The fluid diodes (40) provide for the desired flow direction of the fluids used. Further visible are meandering fluid path type structures (14) containing other fluids, such as washing fluid and label fluid. These fluids are directed 25 towards the pump chamber by means of the pump. Thereto the at least one provision for the outlet (18) is opened, in order to compensate for the volume of fluid moved. The design of the provision for the inlet and the fluid connection towards the reaction chamber limit the back flow. The design of the reaction chamber is such 30 that it provides for optimal flow profiles, with minimised dead volume and optimised contact with the moiety present. Furthermore a large meandering fluid path is present for collecting the waste fluid from the reaction chamber. Also visible are fluid connections (19) between the bottom side and the top side of the microfluidic device.

[0047] The term "label" as used herein refers to a compound that can be detected directly or indirectly by 45 the measuring the device. So it can also be a particle containing a label, such as a 3-dimensional structure with a label inside. Or it may be an enzyme that first may react with another component present. It also can form a bond with the moiety and/or at least one of the components present in the fluid. Preferred one or more of the following typically characterizes labels: high sensitivity, high stability, causing a low background signal upon detection, low environmental sensitivity and high specificity in labelling.

[0048] A preferred embodiment of the microfluidic device further comprises at least one provision for an inlet (15) which is arranged to receive the fluid, said inlet being sealed by a seal from the environment, which seal

is to be removed upon use, thereby opening at least one entrance to the at least one provision for an inlet and/or which comprises at least one provision for an outlet (18) that is prior to use sealed from the environment by a seal, which seal is to be removed upon use. This has the advantage that the microfluidic device has a prolonged shelf life. Typically the at least one provision for an outlet and the least one provision for an inlet will also be opened prior to use, the first to enable a fluid to flow, the latter to enable a sample to be inserted into the microfluidic device. This has the advantage that the microfluidic device has a prolonged shelf life. Furthermore it is ready to use. And it can be used once and then disposed of. As it comprises a minimal amount of fluids the environmental impact is small. A preferred embodiment of the microfluidic device further comprises at least one soft seal that closes at least a part of the recess.

[0049] The term "soft seal" as used herein refers to a seal that closes a part of the recess, thereby preventing liquid and/or gas to flow from this apart to another part. The soft seal is broken upon applying a limited amount of force, such as the pressure or energy transferred by a pump. Typically the soft seal is selected from the group of fluids with relatively high viscosity. Soft seal material can e.g. be selected from the group of silicones and silicone oils. The advantage of such a seal is clearly that it prevents leakage, not only in normal circumstances, but also for instance during transport of the device.

[0050] A preferred embodiment of the microfluidic device characterized in that at least one of the fluid connection(s) is equipped with fluid diodes for resisting a flow of the fluid through the fluid connections in one direction. This fluid diodes (40) are also shown if figure 3. A clear advantage of the use of such diodes is that it directs the flow of fluids in a desired direction, whereas it reduces the flow in the other direction significantly. This has the further advantage that it enables the performance of more complicated reaction programs. Furthermore in these reaction programs or reaction schemes various fluids can be used, whereas without the diodes this would be much more complicated or even impossible. A further advantage is that it allows for much more complex structures on (both sides) of the body, whereby many more fluid connections are made, without the fluids being undesirably mixed or flowed. A further advantage is that recesses and/or chambers that comprise different fluids can be separated if required in one flow direction.

[0051] The term "fluid diode" as used herein refers to a structure within a recess, which is characterized in that the resistance to a fluid flow is significantly larger in one direction compared to the other and which has no moving parts. In other words, the resistance towards a fluid flow changes significantly with change in the direction of the flow. It further has the advantage that applying a different frequency from the actuator of the pump may reverse the preferred flow direction. In a preferred embodiment the fluid diodes have a brush-like or valve-like

structure. The proper orientation of the brush structures is not intuitively obvious even to one skilled in the art. It rather must be determined with mathematical modelling of the fluid flow and by experimentation. Figure 5 shows

5 top view of a preferred embodiment of a fluid diode in a body (11). The recess (14) contains brush like structures (51), which act as a resistance to the flow in one direction. The arrow (53) indicates the flow direction that is not hindered. In that case, the fluid enters the fluid diode indicated with (52). The fluid diode has a width of 1 mm. The brushes are 0,5 mm long and 70 µm wide.

[0052] In a next embodiment of the microfluidic device it further comprises further elements for directing the fluid. Such elements are for instance valves. This has the 15 advantage that even more complex pumping operations and reaction sequences can be performed.

[0053] In yet another embodiment the microfluidic device further comprises a readable information carrier. In 20 a preferred embodiment the readable information carrier is an optically or electrically readable information carrier, most preferably it is an electrically readable information carrier. The information carrier may be detachable from the microfluidic device. As for example in emergency situations a measurement would be performed, 25 thereby using a microfluidic device according to the invention. The result of the measurement may need to be logged into a central computer; therefore the results need to be transferred from the microfluidic device to the computer. The device itself may be contaminated and therefore needs to be disposed. A detachable information carrier thus allows for the desired transfer. The 30 readable information carrier contains data that is for instance relating to the microfluidic device and/or relating to a method of operating the microfluidic device and/or 35 the system for characterising a fluid. It further provides operating instructions, such as pump frequencies. These instructions optimise the use of materials contained in the device, the time necessary to label the components of interest, the accuracy of the result obtained.

40 It also provides measurement instructions, such as a pump times, intervals etc. It also contains data relevant to the device, such as intended use, and it contains data relevant to the measurement, such as type of device and calibration curve respectively. In another embodiment 45 the information carrier just provides the measurement system with the intended use. This reference allows the measurement system then to retrieve and/or calculate the above-mentioned data and use it accordingly. This information present in the information carrier has the advantage that the use of the microfluidic device, in combination with the measurement device is very easy and 50 can be performed by persons with limited skills and knowledge.

[0054] In a further embodiment the microfluidic device 55 further comprises labels. In a preferred embodiment these labels are selected from fluorescent labels, chemiluminescent labels and colorimetric labels.

[0055] In a first embodiment the measurement device

suitable for use in a system characterises a fluid in the microfluidic device of the present invention, which fluid is suspected to comprise at least one component to be characterised or analysed, which measurement device is associated to the microfluidic device Figure 6 represents a schematic layout of the actual detection in the measurement device. A light source (41) is used to radiate (42) a component present in the reaction chamber (16). In the figure the light source is a laser. In order to optimise the radiation a lens (43) may be used. If a component is present in the reaction chamber (16) that emits radiation (45), this radiation can optionally be passed through a filter (46). In the figure it is assumed that the component is or comprises a fluorescent label. If light is emitted it is detected by a detection unit (47).

[0056] In a second embodiment the measurement device further comprises, at least one communication port for transferring data, at least one read-out unit for reading in characteristics of the microfluidic device, at least one light source illuminating the reaction chamber in the microfluidic device, at least one detection element for detecting the radiation emitted from the reaction chamber, an information unit displaying characteristics of the fluid. This measurement has the advantage that it is simple in use in combination with the microfluidic device of the invention and can be performed by persons with limited skills and knowledge. Furthermore the measurement device itself is simple and can be constructed easily. It further contains no expensive elements, which allows for the measurement to be very economical. It further makes use of readily available elements, which makes the manufacture of it easy, economical and reliable. It also has the advantage that the result of a measurement is available within a limited amount of time. Typically a measurement from start to finish takes 1-15 minutes. The results can be transferred to a data-collecting system, such as a computer, using the communication port. The read-out unit allows for the information relating to the microfluidic device and the type of measurement to be transferred to the measuring device and subsequently to the data-collecting device, without any burden. The information safeguards the correct use of the measurement device and therefore also of the results obtained. The unit that is associated with the microfluidic device furthermore provides for a simple to perform measurement.

[0057] The information unit provides for the opportunity to directly obtain a visual result that can be used in a subsequent action, such as treatment.

[0058] The measurement device will typically be used to characterise or analyse a component that is selected from the group consisting an antibody, a cell receptor, an antigen, a receptor ligand, an enzyme, a body, an immunochemical, an immunoglobulin, a virus, a virus binding component, a protein, a cellular factor, hormones, allergens, a growth factor, an cell-inhibitor, DNA, RNA, antigen to be bound to an antibody or receptor or a combination thereof

[0059] In a further embodiment the measurement device further comprises a communication port of an USB-type. This is a standard interface for electronic devices, which allows for easy installation and easy data transfer.

5 And it is economical.

[0060] In another embodiment the measurement device further comprises a chip-reader as the read-out unit. This is a standard interface for reading information on chips, which allows for easy installation and easy data

10 transfer. Typically the read-out unit can also be used to write data on the chip. This data comprises the result or results of the characterisation or analysis. This has the advantage that the information can later on be logged, for instance to a central computer facility. It can

15 further comprise sample information and patient information. And it is economical.

[0061] In a further embodiment the measurement device further comprises a laser as the light source. A laser has the advantage of emitting nearly monochromatic light, though an option may be to use polychromatic (laser) light and use filters. The latter is preferably used in

20 the case that the measurement device has a multipurpose use and/or is used to detect various wavelengths of emitted light at the same time. Also a laser can be easily replaced if necessary. It also is a reliable light source that consumes a low amount of energy.

[0062] In a further embodiment the measurement device comprises a photodiode, a CCD, a photo multiplier tube (PMT) or a series of photodiodes as the detection 30 element. The detection element is capable of detecting the light that is emitted by the at least one component to be characterised or analysed. The advantage of photodiodes or CCD is that they are quite specific with respect to the wavelength chosen.

[0063] In a further embodiment the measurement device further comprises software. The software is used for at least directing the at least one pump to transfer an external pressure to at least one of the chambers of the microfluidic device of the invention. The software further provides for the information on the microfluidic device to be transferred to the measurement device and subsequently to perform the reaction sequence. It provides for the determination of the concentration of the at least one component suspected to be present in the fluid. It therefore provides for a simple operation procedure and minimises the risk for mistakes.

[0064] Also the measurement device may comprise a local memory and/or computing chip, in order to store and retrieve data as well as to perform calculations and to control the other components present.

[0065] For the man skilled in the art it is a routine job to construct such a measurement device out of widely available parts.

[0066] Further the invention describes a method for characterising or analysing characterising or analysing at least one component that is suspected to be present in a fluid comprising,

- a. introducing a fluid to be characterised or analysed in a microfluidic device according to the invention,
- b. moving the fluid to a reaction chamber,
- c. reacting the fluid with the moiety that binds,
- d. moving a washing fluid to the reaction chamber and washing the reaction chamber,
- e. illuminating the reaction chamber to a light source emitting radiation,
- f. detecting the radiation emitted.

[0067] Typically, the method comprises separating a mixture of components, which mixture of components may contain the components of interest. To detect the component of interest, the mixture of components or the separated components are contacted to a component-binding moiety specific to the component of interest. The component-binding moiety binds to the component of interest and is detected, thereby detecting the component of interest, either by measuring the component-bonding moiety directly or by measuring the result of competition with other components, that have been replaced by the component of interest. The embodiment of the present invention has the further advantage that the complete procedure can be performed on one microfluidic device and the measurement result can be obtained directly from the measurement device associated with it.

[0068] In a second embodiment for a method for characterising or analysing characterising or analysing at least one component that is suspected to be present in a fluid comprising,

- a. introducing a fluid to be characterised or analysed in a microfluidic device according to the invention,
- b. moving the fluid to a reaction chamber,
- c. reacting the fluid with the moiety that binds,
- d. moving a label fluid to the reaction chamber and reacting the label with the moiety that binds and/or with the component to be characterised or analysed,
- e. washing the reaction chamber with a washing fluid,
- f. illuminating the reaction chamber to a light source emitting radiation,
- g. detecting the radiation emitted.

[0069] In this embodiment, a component of interest is labelled with a detectable label, subsequently bound to the binding moiety and then detected. The detection signal is then calculated to a concentration, using a calibration curve of the label.

[0070] In a preferred embodiment the microfluidic device is in arrangement with a piezo-pump. The piezo-pump is instructed to perform complicated pumping cycles, involving pumping a fluid, leaving the fluid to react and repeating such steps. In a further embodiment com-

plex pumping cycles of one fluid are alternated with complex pumping cycles of another fluid. For instance, first a label fluid is moved to the reaction chamber with such a pumping cycle and subsequently a wash fluid, which steps are repeated if required. This clearly has the advantage that the reaction in the chamber can be optimised by controlling the amount of fluid moved to the reaction chamber. This is important as the reaction is mainly determined by fluid dynamics. By supplying an amount of fluid each time the fluid dynamics cause exhaustion, the reaction rate is significantly enhanced. Therefore the reaction rate is to a large extend determined by the kinetics of the pumping, rather than by the movement of components in the fluid due to concentration gradients. This improves the reaction time as well as the sensitivity. A further advantage is that the amount of fluid used are minimised with such a procedure. This has the further advantage that even more complicated pumping operations and reaction sequences can be performed.

[0071] In another embodiment the moiety can be present in the form of (magnetic and/or non-magnetic antibody) coated particles. The particles are optionally stacked in a detection region. The component-binding moiety thereby binds to the component of interest, thus providing detection of the component of interest.

[0072] In a further aspect, the method comprises providing a body structure having a plurality of recesses disposed therein, the plurality comprising a microfluidic separation recess and at least one side recess intersecting the separation recess, wherein the separation recess and the side recess are fluidly coupled. A mixture of components is flowed through the separation recess, resulting in separated components. A labelled component-binding moiety is subsequently flown through a side recess and into the separation recess, wherein it binds to the component of interest. The component-binding moiety is then detected, thereby detecting the component of interest.

[0073] In a further embodiment the steps d and e are repeated a number of times.

[0074] This has the advantage that more label (step d) is bound to the moiety and thereby the emitted radiation in the detection steps is increased. Furthermore it allows to optimise the use of label fluid.

[0075] In a further embodiment the method for characterising or analysing at least one component that is suspected to be present in a fluid comprises,

- a. introducing a fluid to be characterised or analysed in a microfluidic device according to the invention,
- b. moving the fluid towards a reaction chamber,
- c. combining the fluid with at least one label fluid forming a combined fluid before the reaction chamber,
- d. reacting the label with the component to be characterised or analysed,

e. moving the combined fluid to the reaction chamber and reacting the label with the moiety that binds,
f. washing the reaction chamber with a washing fluid,
g. illuminating the reaction chamber to a light source emitting radiation, detecting the radiation emitted. This embodiment has the advantage that the label and component to be characterised or analysed mix and react in the fluid flow towards the reaction chamber an further inside the reaction chamber. Thereby the amount of label and/or component reacted is increased, due to improved kinetics in the flow. Further the incubation time is reduced, resulting in a shorter overall measurement time.

[0076] In a next embodiment the characterising or analysing method further comprises the use of a fluorescent or chemiluminescent label.

[0077] The separated components are typically labelled components that are optionally detected simultaneously with the component-binding moiety. This embodiment optionally includes deconvoluting the detection signal to identify the separated components and the component of interest. This embodiment includes two detectably different label moieties having detectably different spectral characteristics, such as different excitation or emission maximum. The different labels include, but are not limited to fluorescent labels, chemiluminescent labels and colorimetric labels. For example, the separated components are optionally labelled with a first fluorescent dye and the component-binding moiety is labelled with a second fluorescent dye. These two dyes are typically detectably different. In another embodiment, the component of interest and the component-binding moiety are optionally labelled with detectably different colorimetric labels. In another embodiment, the component of interest is labelled with one type of label, e.g., chemiluminescent, and the component-binding moiety is labelled with a second type of label, e.g., fluorescent.

[0078] In a further embodiment the characterising or analysing method the component to be characterised or analysed is selected from the group consisting an antibody, a cell receptor, an antigen, a receptor ligand, an enzyme, a body, an immunochemical, an immunoglobulin, a virus, a virus binding component, a protein, a cellular factor, hormones, allergenics, a growth factor, an cell-inhibitor, DNA, RNA, antigen to be bound to an antibody or receptor or a combination thereof. The fluid is preferably a body fluid, such as a blood, serum, urine, saliva, or extracts, such as plant-extracts.

[0079] In a next embodiment the characterising or analysing method further comprises the use of a laser as the light-source.

[0080] The following examples are merely meant to illustrate the invention and are not intended to limit the scope of invention in any way.

Example 1.

[0081] This example describes the measurement of myoglobin concentration in a blood sample.

- 5 [0082] A polymethylmethacrylate (PMMA)-microfluidic device containing Piezo-pumps is cleaned with ethanol (70%) followed by demineralised water. The microfluidic device is completely dried by applying compressed air. A piece of transparent foil with the same size as the microfluidic device (seals the recesses in the PMMA structure from the environment. The foil is from Permacel, a Nitto Denko company). This way the micro-recesses are closed. A window in the foil was cut just over the reaction area, in order to be able to detect the light emitted by the label. The diameter of the window is 3,6 mm, of which 3,5 mm is covered with nitrocellulose. The depth of the chamber is about 450 µm.
- 10 [0083] Strips of polyester supported nitrocellulose (from Whatman) were coated with Monoclonal mouse IgG anti human Myoglobin (Medix, Finland). Spots of 1 µl (1 µg Ab/µl HEPES buffer pH 8) were dropped onto the nitrocellulose. The spots are dried at room temperature for 30 minutes. After this the nitrocellulose strips are blocked with a HEPES buffer + 0.1% Tween 20™
- 15 (from ICI, USA) at pH 8 for one hour. An additional drying step (4 hours at room temperature) is required before the strips are ready to use.
- 20 [0084] The polyester support of the nitrocellulose strip was mounted on a double-sided adhesive tape. After 30 that the nitrocellulose side is covered with a transparent plastic foil to avoid any damage. From these strips dots of 35 mm are prepared by using a revolver punch gripper from Conrad. It should be taken into consideration that the coated antibody is in the centre of the 35 mm dot of nitrocellulose. The protection foil and the double side adhesive tape are removed, and the polyester support side is put in the centre of to a 15x10 mm piece of Permacel foil. This foil is put in the window where the reaction area is located. The nitrocellulose should face the
- 25 PMMA structure, and it is located as close as possible to the outlet of the microrecess. The PMMA reaction area has a diameter of 36 mm. The reaction area is sealed with the Permacel foil containing the piece of nitrocellulose.
- 30 [0085] Using 1 ml syringes, the label and wash reservoirs are filled with their respective buffers. Both reservoirs are consisting of the chamber under the piezo-pump, the fluid diodes and the microrecesses, which are needed for a proper functioning. The label solution is a
- 35 HEPES based buffer (pH8) containing a biotinylated monoclonal antibody (anti Myoglobin) with the fluorescence labelled streptavidin (Molecular Probes). The fluorescence signal is generated by a so-called Fluorescence Resonance Energy Transfer system. When the complex is excited at 635 nm it emits light at 778 nm.
- 40 [0086] After filling the reservoirs, a syringe is filled with sample (Myoglobin Std from SCIPAC (Scipac Ltd. Kent UK) diluted with HEPES buffer pH 8, at a concen-

tration of 0 ng/ml, 100 ng/ml or 1000 ng/ml respectively and connected to the sample inlet from the microfluidic device.

[0087] Piezo pumps are connected to the amplifier and the wash and label syringes were removed from the microfluidic device. At this stage, the microfluidic device was ready-to-use.

[0088] Subsequently the following steps are carried out. The syringe pump injects the sample in with 100 msecounds breaks. After 300 seconds of sample incubation the label fluid pump starts working at 3.5V (times -150) and a 0 offset with a counter pumping of the wash-buffer (amplitude 1.1 V, magnification -150 times), offset = 0, no phase shift). After 4 seconds pumping the label fluid the reaction area is incubated during 75 seconds with the label. The label fluid is refreshed seven times. This is achieved by pumping for 0,5 seconds with a 75 seconds incubation time in between each time. After the last label interval the washing pump starts to work at 3,5 V (times -150) and a 0 V offset with a counter pumping of the label (amplitude 1.0 V (times -150), offset = 0 V, no phase shift). After 5 seconds of pumping the wash-buffer the reaction area is soaked during 15 seconds. The label fluid is refreshed eight times by 0,5 seconds pumping with 15 seconds diffusion in between. The washing interval ends with 15 seconds of diffusion.

[0089] When the program is finished, the openings for the wash, label and waste fluids are sealed with Permacel foil. The sample syringe is removed and the sample inlet is also sealed with Permacel foil. The piezo-pumps are disconnected and the piece of nitrocellulose including the labelled component is removed from the reaction chamber.

[0090] The piece of nitrocellulose is placed in a strip-holder (centre position, always in the same place) of the fluorescence reader from LRE Technology Partner GmbH and kept in the dark. After drying the nitrocellulose for 30 minutes it was read out with the LRE-Reader. The reader excites the fluorescent label with a laser diode that emits at 642 nm, and a photodiode collecting the emitted light from the dye above 725 nm. The slight difference in wavelength is caused by the difference between the theoretical value and the value actually used and/or obtained. When scanning the piece of nitrocellulose the fluorescence scanner obtains one value (in arbitrary fluorescence units) every 0.054 mm.

[0091] In figure 7 the results of the measurement are shown. The first three peaks are three assays done on the same body, which were incubated with 0, 100, and 1000 ng/ml of Myoglobin, successively. The second part of the figure shows a group of three peaks under identical conditions but on another body. The detector determines the peak width. The detector scans the nitrocellulose, each time generating a signal.

[0092] Going from left to right the maximum peak level increases with Myoglobin concentration. As the spots used were placed as a liquid on the nitrocellulose the peaks can be somewhat α -symmetrical. The second se-

ries is somewhat different with respect to peak (height and width) as compared to the first series, which is due to statistical variation and reproducibility.

5 Example 2.

[0093] The microfluidic device according to the present invention is produced by methods known to the person skilled in the art.

[0094] An embodiment according to the invention consists out of a PMMA body (see figure 4). Figure 4 shows the layout, though the dimensions shown herein are different in reality. The size of this body is 60 by 47 by 1 mm³. The recesses in the front side and back side of the body are manufactured by injection-moulding the PMMA body. Also the fluid diodes are made by injection moulding. The width of the fluid connections is about 1 mm, the depth is approximately 450 μ m. The diameter of the reaction chamber is about 4 mm.

[0095] The body consists out of one provision for an inlet. Also three provisions for an outlet are present, the first in connection with the meandering fluid path with the wash fluid, the second in connection with the meandering fluid path for collecting the waste fluid, and the

25 third in connection with the meandering fluid path with the label fluid. It further contains fluid connections between the provision for the inlet and the reaction chamber, between the meandering fluid path for collecting the waste fluid and the reaction chamber, between the meandering fluid path with the wash fluid and the reaction chamber, between the meandering fluid path with the label fluid and the reaction chamber. Also contains two pump chambers, one for pumping the label fluid and

30 one for pumping the wash fluid. These pump chambers are connected to the wash fluid and label fluid by a fluid connection from one side of the body to the other. Further four fluid diodes are present for directing the fluid.

[0096] The cover layers are connected to the body by hot-welding PMMA. Before this hot-welding step a strip 35 of nitrocellulose was place on the covering PMMA layer, in a location that it coincides with the reaction chamber. The reaction chamber contains as a moiety monoclonal mouse IgG anti human Myoglobin.

[0097] This moiety was deposited on the cover layer 40 prior to the hot-welding step. A drop of fluid containing the moiety was dripped on the nitrocellulose strip, of which the location also coincides with the reaction chamber.

[0098] The pumps used are readily available piezo-pumps.

[0099] The wash fluid used is demineralised water. The label fluid solution is a HEPES based buffer (pH 8) containing a biotinylated monoclonal antibody (anti Myoglobin) with the fluorescence labelled streptavidin (Molecular Probes).

Example 3

[0100] A preferred embodiment of the characterising or analysing device is constructed out of components that are readily available.

[0101] The housing of the measurement device can be constructed by injection-moulding. The material used in the housing can be typically a polymer.

[0102] The communication ports used can be standard USB-interfaces, consisting out of USB-plugs and USB-sockets.

[0103] The read-in unit can be a standard chip-read-out unit, used for instance for banking-cards, which is widely available.

[0104] The receiving device can be a standard receiving unit, used for instance for the intake of banking-cards in an ATM, which is widely available.

[0105] Typically the measurement device contains a laser. A preferred laser can be a standard 635 nm laser, which is widely available. The type of laser and frequency used will clearly depend on the component that is suspected to be present and/or the label used.

[0106] The detection unit can be a standard 778 nm detection unit, which is widely available.

[0107] Typical voltage amplitudes applied to the piezo-pump are 150V and 300V. The achieved pressure is from 200 to 4000 Pa, but may vary upon the piezo-pump used, the type of fluid diode and pumping altitude.

[0108] The information unit can be a standard LCD-display, which is widely available.

Claims

1. System for characterising or analysing a fluid, which fluid is suspected to comprise at least one component to be characterised or analysed, comprising a microfluidic device, at least one pump for transporting the fluid and a measurement device which is arranged to characterise or analyse the at least one component in the microfluidic device, which microfluidic device comprises at least one body(11), wherein the body has at least one surface, wherein the at least one surface has at least a part of the recess for containing the fluid in the microfluidic device and/or transporting the fluid in the microfluidic device through at least a part of the microfluidic device, wherein the body has at least one provision for an inlet (15) and at least one provision for an outlet (18), wherein at least a part of said recess (16) is a reaction chamber, which reaction chamber comprises a moiety that binds to the at least one component that is suspected to be present and that is to be characterised or analysed, which reaction chamber is arranged for characterising or analysing the at least one component,

wherein at least a part of said recess is a fluid connection (14) between the at least one provision for an inlet (15) and the at least one provision for an outlet,

5 wherein at least a part of said recess (17) is a pump chamber,

wherein at least the reaction chamber, pump chamber and fluid connection are sealed from the environment by at least one cover layer (12, 13).

2. A system according to claim 1, where the measurement device comprises a measurement device for characterising the fluid, wherein the measurement device is arranged to obtain information based on an optical technique selected from the group consisting of fluorescence, chemiluminescence, time resolved fluorescence, time resolved chemiluminescence, colorimetry or a combination thereof, or from the group consisting of magnetic measurements, resistivity measurements, capacity measurements, surface plasma resonance (SPR) measurements, or a combination thereof.
3. A system according to anyone of claims 1-2, wherein the characteristics of the fluid comprise a concentration of at least one of the components of the fluid.
4. A system according to anyone of claims 1-3, wherein in the at least one pump is a piezo-pump.
5. A system according to anyone of claims 1-4, which is a disposable.

35 6. Microfluidic device arranged for use in the system of anyone of the preceding claims, which microfluidic device comprises at least one body (11), wherein the body has at least one surface, wherein the at least one surface has at least a part of the recess for containing the fluid in the microfluidic device and/or transporting the fluid in the microfluidic device through at least a part of the microfluidic device, wherein the body has at least one provision for an inlet (15) and at least one provision for an outlet (18), 40 wherein at least a part of said recess (16) is a reaction chamber, which reaction chamber comprises a moiety that binds to the at least one component that is suspected to be present and that is to be characterised or analysed, which reaction chamber is arranged for characterising or analysing the at least one component, 45 wherein at least a part of said recess is a fluid connection (14) between the at least one provision for an inlet (15) and the at least one provision for an outlet, 50 wherein at least a part of said recess (17) is a pump chamber,

- wherein at least the reaction chamber, pump chamber and fluid connection are sealed from the environment by at least one cover layer (12, 13).
7. Microfluidic device according claim 6, wherein one cover layer and another cover layer form together one part.
8. Microfluidic device according to anyone of claims 6 or 7, which further comprises a filter in the at least one provision for an inlet.
9. Microfluidic device according to anyone of claims 6-8, further comprising
- a. at least a part of the recess for a washing fluid, and
 - b. at least a part of the recess for collecting waste fluid.
10. Microfluidic device according to anyone of claims 6-9, which further comprises at least a part of the recess which comprises at least one label fluid, which label binds to the moiety in the reaction chamber and/or to the at least one component to be characterised or analysed.
11. A microfluidic device according to anyone of claims 6-10, which comprises at least one provision for an inlet (15) which is arranged to receive the fluid, said inlet being sealed by a seal from the environment, which seal is to be removed upon use, thereby opening at least one entrance to the at least one provision for an inlet and/or which comprises at least one provision for an outlet (18) that is prior to use sealed from the environment by a seal, which seal is to be removed upon use.
12. A microfluidic device according to anyone of claims 6-11, which comprises at least one soft seal that closes at least a part of the recess.
13. The microfluidic device according to anyone of the claims 6-12, **characterized in that** at least one of the fluid connection(s) is equipped with fluid diodes for resisting a flow of the fluid through the fluid connections in one direction.
14. The microfluidic device according to claim 13, wherein the fluid diodes have a brush-like or a valve-like structure.
15. The microfluidic device according to anyone of the claims 6-14, wherein the moiety that binds is chosen from the group consisting of a nuclear receptor, an intracellular receptor, a solubilized receptor, an antibody, an antigen, an enzyme, avidin, a polynucleotide and a polysaccharide.
16. The microfluidic device according to anyone of the claims 6-15, which further comprises elements for directing the fluid.
- 5 17. The microfluidic device according to anyone of the claims 6-16, which further comprises a readable information carrier.
- 10 18. The microfluidic device according to anyone of the claim 10 or claims 11-17 dependent thereon, wherein in the at least one label is selected from the group consisting fluorescent labels, chemiluminescent labels and colorimetric labels.
- 15 19. Measurement device suitable for use in a system according to anyone of claims 1-5, for characterising or analysing a fluid in the microfluidic device of anyone of claims 6-18, which fluid is suspected to comprise at least one component to be characterised or analysed, comprising a characterising or analysing apparatus to be associated to the microfluidic device.
- 20 20. The measurement device according to claim 19, which further comprises,
- 25
- a. at least one communication port for transferring data,
 - b. at least one read-out unit for reading in characteristics of the microfluidic device,
 - c. at least one light source illuminating the reaction chamber in the microfluidic device,
 - d. at least one detection element for detecting the radiation emitted from the reaction chamber,
 - e. an information unit displaying characteristics of the fluid.
- 30 21. Measurement device according to claim 20, wherein the communication port is of an USB-type.
- 35 22. Measurement device according to anyone of the claims 20-21, wherein the read-out unit is of a chip-reader
- 40 23. Measurement device according to anyone of the claims 20-22, wherein the light source is a laser.
- 45 24. Measurement device according to anyone of the claims 20-23, wherein the detection element is a photodiode is, a CCD, a photo multiplier tube (PMT) or a series of photodiodes.
- 50 25. Measurement device according to anyone of the claims 20-24, which comprises software.
- 55 26. Method for characterising or analysing at least one component that is suspected to be present in a fluid

comprising,

- | | | |
|---|----|--|
| a. introducing a fluid to be characterised or analysed in a microfluidic device according to anyone of claims 6-18, | 5 | be characterised or analysed is selected from the group consisting an antibody, a cell receptor, an antigen, a receptor ligand, an enzyme, a body, an immunochemical, an immunoglobulin, a virus, a virus binding component, hormones, allergenics, a protein, a cellular factor, a growth factor, an cell-inhibitor, DNA, RNA, antigen to be bond to an anti-body or receptor or a combination thereof. |
| b. moving the fluid to a reaction chamber, | | |
| c. reacting the fluid with the moiety that binds, | | |
| d. moving a washing fluid to the reaction chamber and washing the reaction chamber, | 10 | |
| e. illuminating the reaction chamber to a light source emitting radiation, | | |
| f. detecting the radiation emitted. | | |
| 27. Method for characterising or analysing at least one component that is suspected to be present in a fluid comprising according to claim 26, which further comprises between steps c) and d), the step | 15 | |
| a. moving a label fluid to the reaction chamber and reacting the label with the moiety that binds and/or with the component to be | 20 | a. at least two intervals of pumping of at least a fluid and a pause time in between pumping. |
| characterised or analysed. | | |
| 28. A characterising or analysing method according to claim 27, wherein steps d and e are repeated a number of times. | 25 | |
| 29. Method for characterising or analysing at least one component that is suspected to be present in a fluid comprising, | 30 | |
| a. introducing a fluid to be characterised or analysed in a microfluidic device according to anyone of claims 6-18, | 35 | |
| b. moving the fluid towards a reaction chamber, | | |
| c. combining the fluid with at least one label fluid forming a combined fluid before the reaction chamber, | | |
| d. reacting the label with the component to be characterised or analysed, | 40 | |
| e. moving the combined fluid to the reaction chamber and reacting the label with the moiety that binds, | | |
| f. washing the reaction chamber with a washing fluid, | 45 | |
| g. illuminating the reaction chamber to a light source emitting radiation, | | |
| h. detecting the radiation emitted. | 50 | |
| 30. A characterising or analysing method according to anyone of claims 26-29, wherein the label is fluorescent, colorimetric or chemiluminescent. | | |
| 31. A characterising or analysing method according anyone of the claims 26-30, wherein the light-source is a laser. | 55 | |

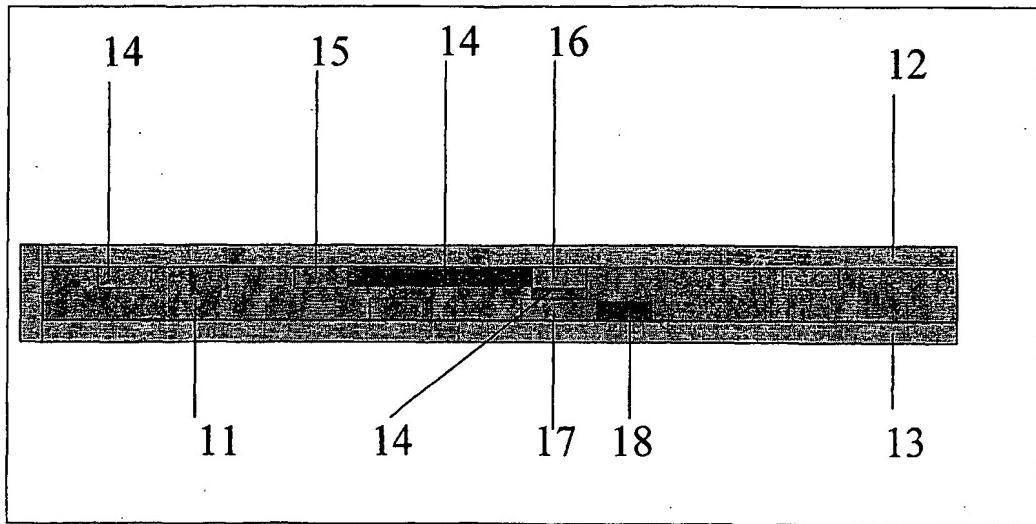


Figure 1

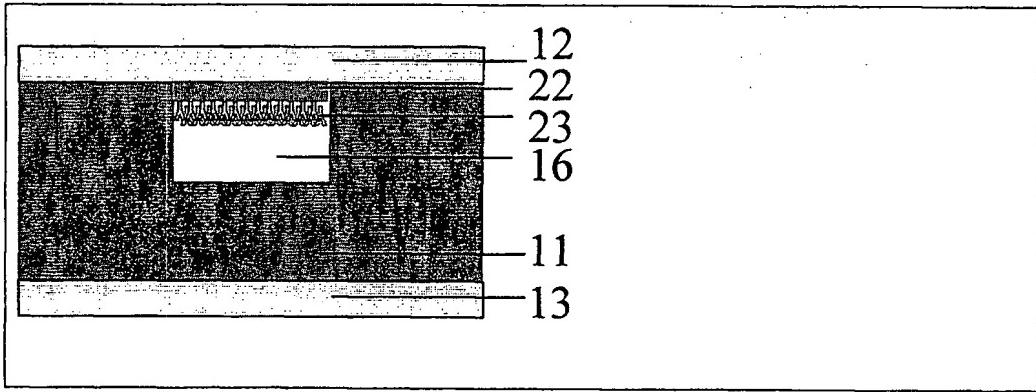


Figure 2

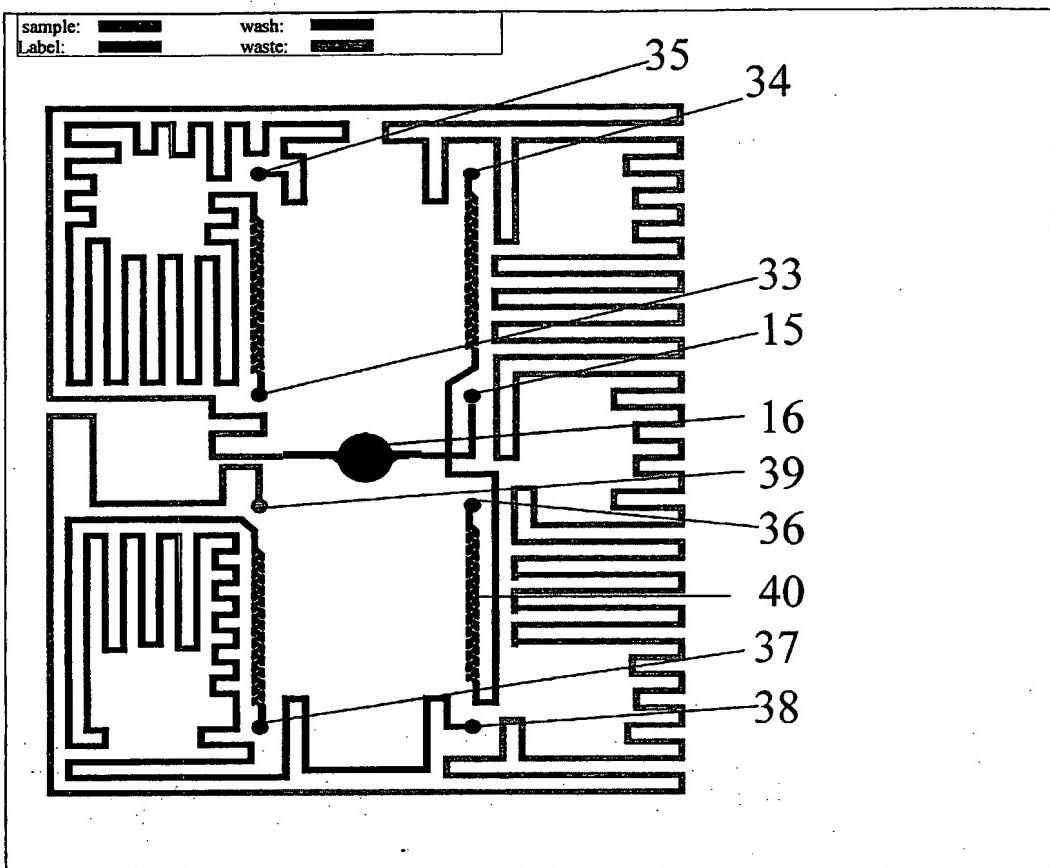


Figure 3

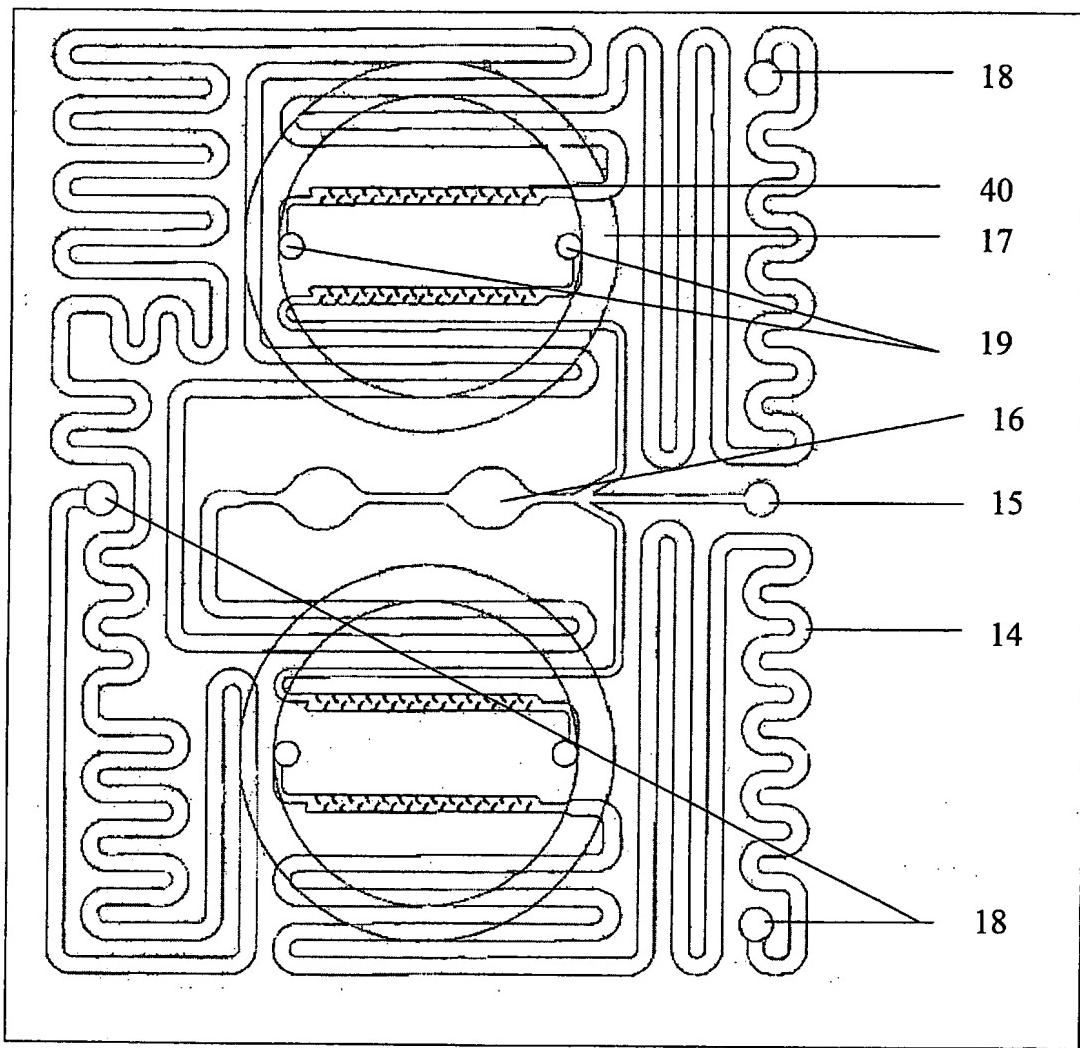


Figure 4

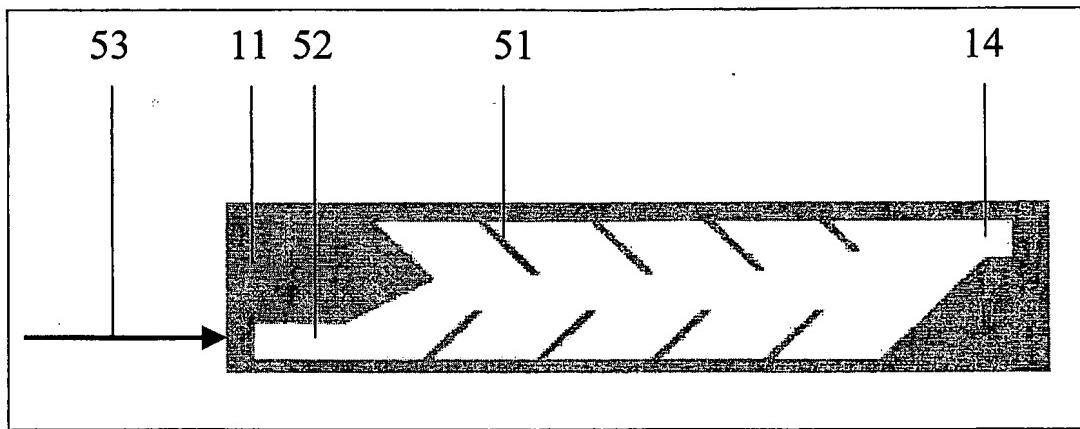


Figure 5

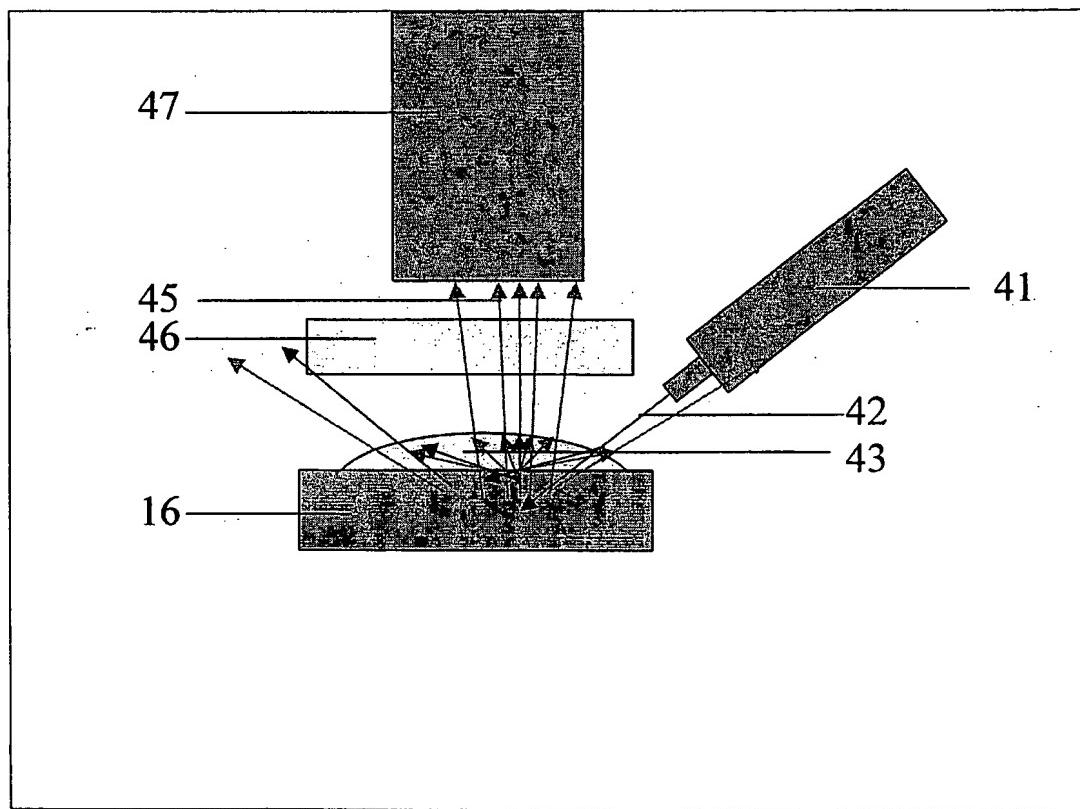


Figure 6

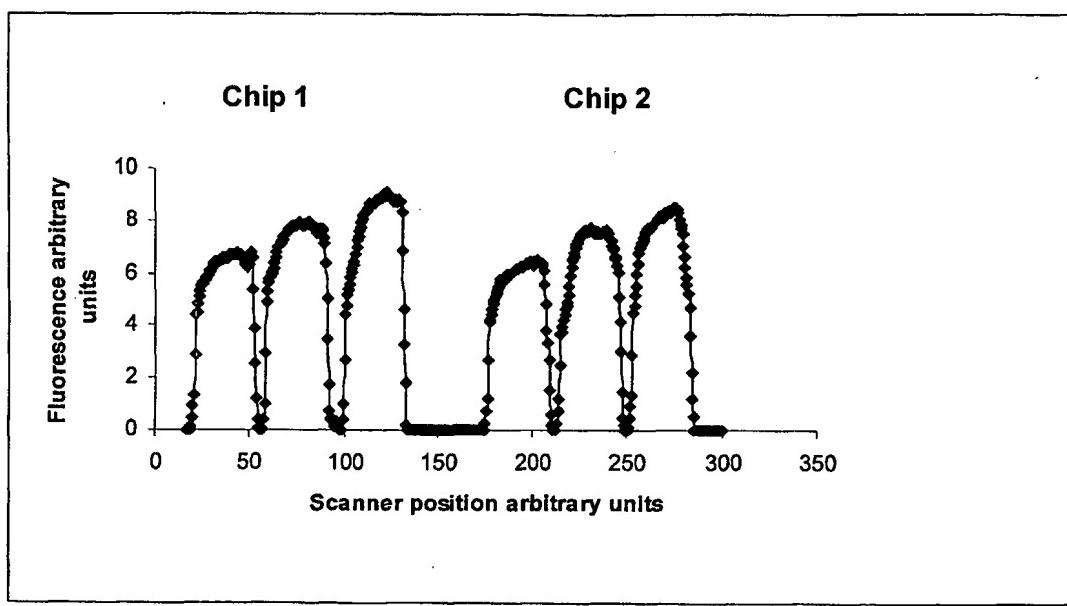


Figure 7



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 04 07 5257

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|--|--|--|--|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.7) |
| X | US 2002/123059 A1 (HO WINSTON Z) 5 September 2002 (2002-09-05) * paragraph [0020] - paragraph [0032] * * abstract * * claims * * figures * ----- | 1-7, 9-12, 15-20, 23-32 | B01J19/00 B01L3/00 |
| | | | TECHNICAL FIELDS SEARCHED (Int.Cl.7) |
| | | | B01J B01L G01N |
| 2 The present search report has been drawn up for all claims | | | |
| Place of search Munich | | Date of completion of the search 29 June 2004 | Examiner Smith-Hewitt, L |
| CATEGORY OF CITED DOCUMENTS | | T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document | |
| X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document | | | |



European Patent
Office

Application Number

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

- All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:



The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. claims: 1-7,9-12,15-20,23-32

a microfluidic device comprising at least one body wherein the body has at least one surface wherein the at least one surface has at least a part of a recess for containing the fluid in the device and transporting the fluid in said device through at least a part of said device wherein the body has at least one provision for an inlet and at least one provision for an outlet wherein part of the recess is a reaction chamber, which comprises a moiety that binds to the component(s) suspected to be present and to be characterised or analysed, the reaction chamber is arranged for characterising or analysing the component, at least a part of the recess is a fluid connection between the at least one provision for an inlet and the at least one provision for an outlet, at least a part of the recess is a pump chamber and the reaction chamber, pump chamber and fluid connection are sealed from the environment by at least one cover layer. System and method using the device. Measurement device for said microfluidic device.

2. claim: 8

A microfluidic device comprising a filter in the provision for an inlet.

3. claims: 13, 14 (e.g. when dependent on claims 6 and 7)

The fluid connection(s) on a microfluidic device are equipped with fluid diodes

4. claim: 21

The communication port of a measurement device is a USB port

5. claim: 22

The read out unit is a chip reader

6. claims: 33,34

a method of pumping comprising the step of at least two intervals of pumping of at least a fluid and a pause time in between pumping.

ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.

EP 04 07 5257

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

29-06-2004

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| US 2002123059 A1 | 05-09-2002 | NONE | |

EPO FORM EP0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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DEMANDE DE BREVET D'INVENTION

A1

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(30) Priorité :

(43) Date de la mise à disposition du public de la demande : 13.09.91 Bulletin 91/37.

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(60) Références à d'autres documents nationaux apparentés :

(71) Demandeur(s) : INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE — FR et SOCIETE NATIONALE ELF AQUITaine — FR.

(72) Inventeur(s) : Ozil Jean-Pierre.

(73) Titulaire(s) :

(74) Mandataire : Cabinet Regimbeau Martin Schrimpf Warcoin Ahner.

(54) Dispositif de culture de cellules assurant leur immobilisation.

(57) La présente invention concerne un dispositif de culture de cellules constitué d'une enceinte comportant une arrivée de fluide et une sortie de fluide, caractérisée en ce qu'à la partie inférieure de l'enceinte, se trouvent un ou plusieurs orifices dont la géométrie est telle qu'elle s'oppose au passage des cellules et en ce que le fluide entrant dans l'enceinte est prélevé, au moins en partie, à travers les orifices, en créant une dépression telle qu'elle assure sensiblement le blocage des cellules sur le ou les orifices, une partie du fluide étant évacuée par débordement.

Ce dispositif peut comporter en outre des électrodes sur la paroi interne de l'enceinte.

La présente invention se rapporte également à un procédé de stimulation artificielle de cellules utilisant ce dispositif.

FR 2 659 347 - A1



La présente invention concerne un dispositif de culture de cellules assurant leur immobilisation, permettant leur traitement par différents milieux en évitant leur manipulation, et un procédé de stimulation de ces cellules utilisant ce dispositif.

5 La présente invention se rapporte également à la stimulation de cellules, en particulier d'ovocytes ou d'oeufs fécondés, par un mécanisme biochimique régulé dans les conditions physiologiques par un oscillateur interne.

10 Elle se rapporte donc à la procédure de clonage des embryons d'animaux et, en particulier à l'obtention d'ovocytes receveurs compétents pour la greffe d'un noyau cellulaire dans un état homogène.

Le clonage d'embryons d'animaux domestiques est la voie permettant de remédier à la variabilité génétique induite par la fécondation et de standardiser les améliorations génétiques d'une race.

15 Lors de la fécondation, l'entrée du spermatozoïde va avoir deux grandes fonctions :

- apport du génome haploïde mâle,
- activation du développement qui restructure le noyau mâle par le cytoplasme de l'ovocyte et favorise les interactions noyau-cytoplasme.

20 Il s'écoule en moyenne 12 à 20 heures entre la fécondation et la première division cellulaire, pendant lesquelles un ensemble de phénomènes ont lieu, les deux génomes paternel et maternel ayant des rôles complémentaires pour un développement ultérieur de l'embryon (Surani et al. 1984, Nature 308, 548-550), mais on n'en connaît pas le mécanisme exact.

25 Le clonage d'un embryon est une méthode qui vise à obtenir le plus d'animaux vivants à la suite du transfert des noyaux cellulaires de cet embryon (qui contient plusieurs cellules) dans des ovocytes enucleés et activés. Les deux fonctions de la fécondation sont dissociées. Afin d'obtenir un développement ultérieur de l'oeuf, il faut procéder à une activation de l'ovocyte receveur avant la greffe nucléaire. L'activation peut aussi s'appliquer aux premières heures du développement de l'oeuf fécondé.

On sait que les oeufs de mammifères peuvent être activés artificiellement par différents stimuli physiques ou chimiques, qu'il s'agisse

de chocs électriques, thermiques ou osmotiques, d'enzymes ou d'agents anesthésiques (Kaufman MH 1983 - Early mammalian development - parthenogenetic studies - Cambridge University Press). Cependant, l'activation est toujours identifiée à un stimulus unique, limité dans le temps, supposé mimer la pénétration du spermatozoïde dans l'oeuf. Aucun de ces traitements ne reproduit la série de changements physiologiques se produisant dans l'oeuf après la pénétration du spermatozoïde.

On ne connaît que quelques cas d'activation parthénogénétique de l'ovocyte de vache (Menezo et al. 1976 - Commission of the European Communities, Agricultural research seminar, Egg transfer in Cattle. Eur 5491) ; aucune méthode vraiment fiable et précise d'activation des ovocytes de bovins n'est disponible. L'activation expérimentale par l'éthanol présente de nombreux inconvénients : grande variabilité des résultats en fonction de l'âge des ovocytes (Cuthbertson, 1983 ; J. Exp. Zool. 226, 311-314).

En ce qui concerne le clonage d'embryons, les premiers résultats incontestés ont été obtenus par S. Willadsen en 1986 chez la brebis. Ils ont été suivis par ceux de Prather et al en 1987 chez la vache, et en 1989 chez la truie. Chez la lapine, les premiers individus issus de clonage ont été obtenus par Stice et Robl en 1988. Les taux de succès de ces expériences rapportés dans les publications ne dépassent généralement pas 4 %. Toutefois, des sociétés américaines (Granada Genetics, Houston, Texas) ou Canadienne (Alta Genetics, Callgary, Alberta), semblent maîtriser la procédure de clonage chez les bovins. Il est dit qu'une centaine de veaux aurait déjà été obtenue sur le continent nord-américain. Cette prise en main industrielle reflète l'intérêt que suscite le clonage d'embryon chez les bovins. Mais les techniques ont-elles vraiment progressé ?

Les procédures décrites dans les publications se ressemblent. Les ovocytes utilisés ont subi une période de vieillissement de 6 à 10 heures. Ce vieillissement est rendu nécessaire pour favoriser l'activation (aucune technique classique d'activation ne permet d'activer des ovocytes fraîchement ovulés même chez les bovins (Ware et al. 1989). Les chromosomes organisés sur la métaphase II sont prélevés en "aveugle" dans

la région du premier globule polaire, mais des techniques de visualisation des chromosomes par fluorescence sont mises en oeuvre. Une cellule embryonnaire provenant d'une morula est introduite sous la zone pellucide et la fusion est obtenue par des impulsions de champs électriques.

5 L'activation de l'ovocyte est généralement provoquée par la procédure de fusion cellulaire ; dans certains cas, elle n'est pas décrite. Deux chercheurs ont pu obtenir un agneau à la suite du transfert dans un ovocyte d'un noyau provenant d'une cellule du bouton embryonnaire (Smith & Wilmut, 1989).

10 Toutefois, le niveau des résultats et la complexité des mécanismes en jeu tels que le stade de différenciation du noyau, la phase du cycle cellulaire, l'état de l'ovocyte receveur, son âge, la procédure d'activation ne permettent pas de comprendre pourquoi certains embryons se développent et d'autres non. De nombreuses théories pour expliquer les échecs comme celle fondée sur l'activité de transcription du noyau transplanté ont été contredites par quelques résultats expérimentaux.

15 Des données nouvelles sont apparues sur les mécanismes physiologiques déclenchés par la fécondation et notamment en ce qui concerne les rythmes des variations de niveau du calcium libre et des seconds messagers comme l'Inositol (1,6,5)-tri-phosphate (InsP3) (Cuthbertson et al. 1981 ; Cuthbertson & Cobbold, 1985 ; Miyazaki et al. 1986 ; Miyazaki, 1988). L'activité périodique de ces seconds messagers pourrait réguler les synthèses des acides nucléiques (ADN et ARNs) et des protéines (Basset et al. 1968 ; Rodan et al. 1978).

20 Le processus est déclenché par le spermatozoïde dans les secondes qui suivent la fécondation. La chaîne de réactions qui aboutit à la production d'InsP3 semble dépendre d'un influx d'ions calcium. L'InsP3 se lie à un récepteur spécifique qui contrôle l'activité d'un canal calcique situé sur la membrane intracellulaire d'un réservoir intracellulaire de calcium. Le calcium ainsi libéré active des protéines spécifiques, qui elles-mêmes activent des processus spécifiques, par exemple la complexation calcium-calmoduline, l'activation de kinase etc. Le calcium est considéré comme l'activateur principal du métabolisme. Ce cycle de réactions se reproduit à

une fréquence de l'ordre de la minute durant plusieurs heures (McCulloh et al. 1983 ; Igusa & Miyazaki, 1983-1986 ; Miyazaki et al. 1986 ; Miyazaki, 1988).

5 Cette activité rythmique, due à un système de signaux intracellulaires émis à une fréquence propre, semble dépendre de la présence de pronuclei mâle et femelle car elle n'a pu être observée sur des oeufs parthénogénétiques activés par des méthodes classiques (Cuthbertson et al. 1981 ; Cuthbertson & cobbold, 1985 ; Miyazaki, 1988).

10 L'injection dans des ovocytes d'une protéine G (guanosine -5'-O-(3-thiotriphosphate) (GTP [S]) induit plusieurs cycles de libération de calcium, mais ne permet pas de maintenir cette activité au-delà du quatrième (Swann, Igusa & Miyazaki, 1989).

15 Les contributions génétiques respectives de l'ovocyte et du spermatozoïde sont très documentées chez la souris. Ces études montrent que le développement normal à terme d'un embryon dépend de la présence permanente durant tout le premier cycle cellulaire des deux pronuclei parentaux (PN) (Surani & Barton 1983 ; McGrath & Solter, 1984 ; Surani et al. 1986 ; Mann & Lovell - Badge, 1986, 1988).

20 La production de jeunes par clonage ne peut être réalisée dans la pratique que si l'on dispose de procédés fiables et reproductibles. La qualité de l'activation de l'oeuf joue dans le développement un rôle important jusque là ignoré.

25 La maîtrise de l'activation comprise comme phénomène rythmique s'étendant sur tout le premier cycle cellulaire est essentielle, non seulement pour produire en routine des ovocytes activés et synchrones mais aussi pour faire bénéficier des oeufs "clonés" de ce type d'activation imposée pour se développer normalement.

30 La présente invention concerne donc un procédé de stimulation artificiel à long terme d'un ensemble de cellules reproduisant l'effet d'un mécanisme de régulation biochimique, contrôlé dans les conditions physiologiques naturelles par un oscillateur interne, caractérisé en ce qu'il comprend les étapes suivantes :

- a) les cellules sont placées en culture in vitro, pendant un temps déterminé,
- b) on effectue un lavage des cellules par le milieu d'impulsion,
- c) les cellules sont placées dans le milieu d'impulsion,
- 5 d) on soumet les cellules à une impulsion générée par un champ électrique,
- e) les cellules sont à nouveau placées dans le milieu de culture,
- f) on répète les étapes précédentes un certain nombre de fois, et
- 10 g) on obtient les cellules dans un état activé homogène.

15 Ce procédé de stimulation bien qu'il puisse être utilisé dans un grand nombre de procédé, a été mis au point dans le cadre de l'activation des ovocytes en vue du clonage d'embryons. Des études récentes ont en effet montré que dans les oeufs de mammifères, la fécondation est accompagnée d'une augmentation transitoire de la concentration intracellulaire de calcium libre ($[Ca^{2+}]_i$), suivie d'une série d'oscillations de cette concentration, qui dure au moins 4 heures.

20 On ignore encore par quel moyen le spermatozoïde déclenche ces variations de $[Ca^{2+}]_i$, ainsi que les fonctions biologiques exactes de ces oscillations de Ca^{2+} de longue durée. Cependant, elles semblent caractéristiques des oeufs fécondés et n'ont jamais été observées quand les ovocytes sont artificiellement activés (Miyazaki 1988 - J. Cell. Biol. 106, 345-353).

25 Le procédé développé dans la présente invention va donc permettre de stimuler périodiquement les ovocytes par des influx de calcium au niveau de la membrane plasmique.

En effet, en introduisant dans le milieu d'impulsion des ions Ca^{2+} , ceux-ci vont pouvoir pénétrer dans la cellule par l'électroporation, c'est-à-dire la création de "pores" générés par l'impulsion électrique.

30 Au-delà des phénomènes immédiats provoqués dans l'ovocyte comme l'expulsion du globule polaire, les effets de cette stimulation se manifesteront sur les stades ultérieurs du développement de l'embryon, alors même que le traitement a cessé. C'est ainsi que les phénomènes de compaction et de cavitation pourront être affectés.

5 Ce procédé peut s'appliquer aux ovocytes fraîchement ovulés, obtenus par stimulation hormonale de la femelle. L'activation des ovocytes dans les conditions définies de fréquence, d'intensité et de durée provoque l'expulsion du deuxième globule polaire. On peut alors prélever les chromosomes qui se trouvent en fin de télophase juste sous le deuxième globule polaire. On dispose d'ovocytes dans le même état physiologique possédant un pronucleus.

10 On introduit alors un blastomère sous la zone pellucide et on procède à la fusion cellulaire par action d'un champ électrique.

15 Le procédé peut alors s'appliquer aux embryons clonés ainsi obtenus, afin d'améliorer leur développement, c'est-à-dire de régulariser les divisions cellulaires et d'obtenir la compaction.

20 La stimulation par ce procédé peut s'appliquer à des ovocytes fraîchement ovulés placés en présence de cytochalasine B, qui inhibe l'expulsion du deuxième globule polaire, maintenant un état diploïde. On obtient ainsi une population d'embryons parthenogénétiques, qui pourront être implantés dans des femelles receveuses et présenteront un développement synchrone du foetus et des annexes embryonnaires.

25 Ces expériences d'abord menées sur des ovocytes de lapine ont été confirmées sur des ovocytes de souris, réputés réfractaires à l'activation juste après l'ovulation. Les performances du procédé sont donc reproductibles d'une espèce à l'autre. La méthode d'activation pourra certainement s'appliquer à des ovocytes fraîchement ovulés de bovin ou d'ovin.

30 Si l'on soustrait les ovocytes en cours de traitement, ils régressent et on obtient certains artefacts, comme des ovocytes en métaphase III reproductibles à volonté, et qui offrent de nouvelles possibilités d'études, notamment sur la dynamique du cytosquelette et l'activation du génome.

35 Le procédé a été conçu sur la base des effets des champs électriques sur les membranes plasmiques.

Il a été montré (Zimmermann, 1982) que des impulsions de champs électriques de l'ordre de 1 à 3 kVcm⁻¹ et d'une durée de quelques μs peuvent créer des micropores dans la membrane plasmique et établir une

communication directe entre les milieux intra et extra cellulaire. Il a été ainsi possible par exemple de faire pénétrer du calcium dans des ovocytes d'oursin en les exposant à des impulsions de champs électriques en présence d'ions calcium (Rossignol et al 1983). L'amplitude de ces influx peut être 5 modulée par la durée de l'impulsion de champ électrique.

Le procédé implique la conjugaison d'une méthode de culture in vitro d'un lot d'ovocytes (ou embryons) par perfusion entre deux électrodes et d'une méthode de lavage avec une solution non conductrice contenant du calcium 0 à 20 µM dans laquelle sont délivrées les impulsions. 10 La présence d'une solution très faiblement conductrice au moment d'une impulsion permet de faire apparaître un champ électrique entre les électrodes. Une présence excessive d'ions diminue l'effet "champ électrique".

15 L'alternance d'une période de culture et d'une période de lavage permet de soumettre fréquemment les ovocytes à des stimulations dans un milieu ionique très bien défini.

20 Ce procédé peut s'étendre à la stimulation des cellules par toute une gamme de signaux simples ou complexes ioniques ou moléculaires à des concentrations variables qui seront déterminés par la composition du milieu d'impulsion.

Ce procédé permet de moduler la fréquence du signal par la durée entre deux lavages, et son amplitude par la durée de l'impulsion électrique.

25 Il est impératif de remplacer totalement le milieu de culture par la solution d'impulsion, car l'intensité du courant due à des excès d'ions provenant du milieu de culture détruirait les ovocytes. Juste après l'impulsion, la solution d'impulsion est remplacée par le milieu de culture, les ovocytes ne pouvant pas survivre dans la solution d'impulsion.

30 Ce procédé peut être piloté par l'intermédiaire d'un logiciel qui permettra de créer des traitements de stimulation selon des équations spécifiques, exponentielles, sinusoïdales, suites de Fourier ou autres.

L'un des aspects de la présente invention est le traitement simultané d'un ensemble d'ovocytes, qui seront ainsi obtenus, activés dans le même état physiologique. Les greffes de noyau pourront ainsi être réalisées sur des ovocytes physiologiquement identiques.

Lors du clonage, la séquence et les phases du cycle cellulaire durant lesquelles sont enchaînées les opérations ont des conséquences très importantes sur le remodelage des noyaux et sur le développement ultérieur.

5 Il est extrêmement intéressant de pouvoir standardiser les différentes phases du traitement des ovocytes, afin d'obtenir une bonne reproductibilité du procédé.

10 La présente invention concerne donc un système dynamique, fonctionnant en continu et pouvant être automatisé, réalisé par un dispositif permettant la succession automatique des étapes de lavage, de stimulation et l'acquisition des paramètres de stimulation.

Ce dispositif est caractérisé par le fait qu'il assure l'immobilisation des cellules pendant les différentes phases du traitement.

15 Il est constitué d'une enceinte comportant une arrivée de fluide et une sortie de fluide et, est caractérisé en ce qu'à la partie inférieure de l'enceinte se trouvent un ou plusieurs orifices dont la géométrie est telle qu'elle s'oppose au passage des cellules et en ce que le liquide entrant dans l'enceinte est prélevé, au moins en partie, à travers les orifices, en créant une dépression telle qu'elle assure sensiblement le blocage des cellules sur le ou les orifices, une partie du fluide étant évacuée par débordement.

20 Ce dispositif permet de retenir les cellules sans les abimer ni introduire une contrainte mécanique parasite. En outre, il permet à tout moment de replacer, prélever ou déplacer des cellules. Ce dispositif permet donc de placer successivement les cellules dans des milieux de composition chimique de différentes natures, durant des périodes et selon une fréquence variables.

25 L'enceinte comporte de préférence sur ses parois internes deux électrodes parallèles, et les cellules sont alignées à égale distance des électrodes, baignant dans le milieu correspondant à la phase du traitement en cours.

30 Grâce à ce dispositif, on peut à volonté cultiver les cellules ou bien dans le milieu d'impulsion faire pénétrer dans la cellule traitée, un ion, une molécule, une substance chimique ou biologique complexe.

Ces milieux, aussi bien le milieu de culture que le milieu d'impulsion, sont en circulation continue, et c'est ce flux de liquide qui par effet de succion assure le maintien des ovocytes entre les électrodes pendant les renouvellements du milieu.

5 Les compositions des milieux de culture sont connues et dépendent des cellules en culture. Le milieu d'impulsion est généralement constitué d'un milieu non ionique pour assurer un effet de champ. Il s'agira d'une solution isotonique par exemple de glucose.

10 La présence d'une grande quantité d'ions peut gravement nuire aux cellules lors de l'impulsion. Il est donc nécessaire de laver les cellules avec le milieu d'impulsion pour éliminer les ions contenus dans le milieu de culture.

15 Ce dispositif peut être conçu comme une chambre d'activation, dont la capacité peut être adaptée au nombre de cellules que l'on souhaite traiter simultanément.

20 Les caractéristiques du procédé mettant en oeuvre le dispositif, peuvent varier largement et ne sont en général limitées que par des conditions telles que les durées de lavage. Ainsi, la fréquence des impulsions est limitée par la durée de lavage minimum par le milieu d'impulsion.

Les paramètres du procédé et du dispositif c'est-à-dire les arrivées et les sorties de fluide, de même que la fréquence, la durée et l'intensité des impulsions électriques arrivant dans les électrodes sont générées par un système électronique.

25 Les caractéristiques des impulsions électriques en elles-mêmes peuvent varier et dépendre des cellules et des buts recherchés.

En général, les champs électriques varient de 1 à 3 Kv. cm⁻¹ et l'impulsion électrique a une durée de 10 µs à 2 000 µs.

30 L'organisation des tubulures qui débouchent dans la chambre permet d'injecter des milieux sans que les ovocytes soient décrochés par les bulles de gaz qui finissent par se former dans les canalisations. De plus, la structure de distribution des milieux permet de limiter considérablement la contamination ionique du milieu de stimulation par les ions du milieu de culture.

Les pompes d'injection des milieux peuvent être commandées par un microordinateur qui déterminera les séquences de lavage répétables tout en maintenant les cellules entre les électrodes.

5 Ce dispositif pourra s'appliquer au traitement des cellules par toute substance que l'on souhaite faire pénétrer dans l'espace intracellulaire. La composition du milieu d'impulsion déterminera la pénétration de cette substance selon un gradient de concentration.

10 Lors d'une culture cellulaire, on pourra provoquer, à un moment déterminé, qui peut être unique, l'entrée dans l'ensemble des cellules de la substance souhaitée. Pour cela on remplacera le milieu de culture par le milieu d'impulsion et on déclenchera l'impulsion de champ électrique, la durée de l'impulsion déterminant la quantité de substance qui pénètre. Ce milieu d'impulsion pourra alors être évacué et les cellules remises en culture dans un milieu approprié. L'avantage de ce système est
15 qu'il permet de traiter simultanément un ensemble de cellules qui sont ainsi synchronisées. Il fonctionne en continu et évite toute manipulation des cellules, assurant ainsi une meilleure reproductibilité et une plus grande rapidité d'exécution. Ceci permet une standardisation des conditions et facilite le transfert de technologies.

20 On peut envisager de faire pénétrer dans les cellules un ion ou une molécule simple. Ce dispositif peut aussi s'appliquer à la stimulation des cellules par une molécule plus complexe, ou à la pénétration de fragments d'ADN, ou d'épisome.

25 Ce dispositif sera utilisé pour réaliser une stimulation cyclique des cellules, plusieurs séquences de base étant réalisées successivement, l'intensité, la durée et la fréquence des impulsions pilotées par l'intermédiaire d'un microordinateur.

Ce dispositif permet la réalisation du procédé d'activation et de synchronisation d'ovocytes, par des stimulations répétées par Ca^{2+} , en vue du clonage d'embryons d'animaux domestiques.

5 Un mode de réalisation de la chambre est représenté en figure 1.

La figure 2 représente les courbes de durée des impulsions pour les différents traitements appliqués aux ovocytes.

10 Dans la figure 1, les ovocytes 1 sont placés sur une fente rectiligne 4, réalisée par la juxtaposition de deux plaques de verre 3 et 5, dont l'écartement est inférieur au diamètre des ovocytes. Les milieux, milieu de culture et milieu d'impulsion, arrivent dans la partie supérieure de la chambre chacun par une tubulure distincte, respectivement 10 et 11, évitant une contamination réciproque.

15 Le milieu 9 correspondant à la phase de traitement en cours arrive en continu et est évacué par une tubulure 6 située à un niveau plus bas que celui des ovocytes. Ce courant de liquide maintient par succion les ovocytes plaqués sur le fond de la chambre.

20 Sur les parois internes de la chambre se trouvent deux électrodes de platine 2 et 7, parallèles, de 1 cm de long.

L'enceinte 8 est thermostatée à 38°C.

Chaque milieu arrive dans la chambre après passage par un système retenant les bulles de gaz de taille importante.

25 Ce système est constitué d'un ballon de verre dans lequel le liquide arrive. De ce ballon part une tige d'or perforée de trous de petit diamètre : après passage à travers cette tige, les bulles de gaz de taille importante sont retenues et le liquide arrive dans la chambre par une tubulure courte.

30 Des lapines ont été superovulées par injection de FSH et de LH, et les ovocytes prélevés dans les oviductes. Après traitement 5 mn par la hyaluronidase, les ovocytes sont placés dans la chambre d'activation, dans le milieu B2 (Menezo, 1976 - C. Hebd. Seanc. Acad. Sci. Paris 282, 1967-1970) à 38°C, dans une atmosphère à 5 % de CO_2 .

Les ovocytes peuvent immédiatement être soumis au traitement d'activation, aucune période de vieillissement n'est nécessaire.

Ils sont soumis pendant 90 minutes à une série d'impulsions de champ électrique, à un rythme d'une impulsion toutes les 4 minutes, soit 22 impulsions de durée décroissante dissipant une énergie totale 1250 µjoules, d'une durée totale de 14 868 µs, le champ électrique a une valeur de 1,8 5 Kv. cm⁻¹.

Avant chaque impulsion, le milieu de culture est remplacé par un milieu d'impulsion constitué d'une solution isotonique de glucose contenant CaCl₂ 10µM.

A la fin du traitement d'activation, tous les ovocytes ont deux 10 globules polaires bien formés. On obtient donc au même moment, à quelques minutes près, une population homogène d'ovocytes activés dans le même état physiologique. Cela présente deux atouts : le premier est de pouvoir toujours réaliser les greffes de noyau dans des oeufs physiologiquement identiques, le second, plus pratique, est de pouvoir opérer au 15 moment où les chromosomes (haploïdes) se trouvent tous en fin de télophase juste sous le deuxième globule polaire. Ce repérage naturel de l'endroit où se trouvent les chromosomes facilite leur retrait en "aveugle" et permet, sans avoir recours à des techniques de marquage fluorescent, d'enchaîner rapidement les étapes de manipulations.

On peut alors effectuer le prélèvement des chromosomes. On remarque que les deux globules polaires ne sont pas toujours accolés (certains sont à l'opposé l'un de l'autre, c'est pourquoi le premier globule polaire n'est pas un bon marqueur de l'endroit où se trouvent les chromosomes maternels). L'efficacité de cette opération, vérifiée par la présence 25 ultérieure d'un pronucleus, est supérieure à 80 %. Un blastomère est ensuite introduit sous la zone pellucide. Quinze à 20 ovocytes peuvent être manipulés en 1 heure.

On procède ensuite à la fusion cellulaire. La méthode de fusion cellulaire dérive des travaux de Zimmerman sur les champs électriques. La procédure a été affinée. (Ozil et Modlinski 1986, J. Embryol. Exp. Morph. 96, 211-228).

On obtient 100 % de fusion. Il sera possible de réaliser automatiquement, sous le contrôle d'un logiciel, l'alignement et la fusion cellulaire dans la chambre de stimulation. Cette procédure permettra de limiter la durée des manipulations et de standardiser les conditions expérimentales.

MATERIELS ET METHODES

On a induit une superovulation chez les femelles de lapin sexuellement matures d'espèces variées par injection sous-cutanée 5 d'hormone stimulant le follicule (FSH) et d'hormone lutéinisante (LH) en accord avec la technique décrite par Kennely et Foote (1965) et modifiée par Thibault (communication personnelle). Les femelles ont reçu 2 mg de FSH en cinq injections à 12 heures d'intervalle : 0,250, 0,250, 0,650, 0,650 et 0,250 mg. 12 heures plus tard, avant la saillie par un mâle vasectomisé, 10 on leur a injecté 0,33 mg de LH.

Les ovocytes ont été récupérés à partir des oviductes 12-15 h après la saillie par lavage par une solution saline de phosphate tamponné (PBS). Ils ont été incubés pendant 5 minutes dans l'hyaluronidase (300 i.u.ml⁻¹ dans du PBS) pour éliminer les cellules folliculaires. Après le 15 traitement, les ovocytes ont été cultivés à 38° dans du milieu B2 (Ménézo 1976) dans une atmosphère à 5 % de CO₂.

Méthode et procédure expérimentale

La perméabilité membranaire des ovocytes de lapin fraîchement ovulés a été transitoirement augmentée par ouverture des pores par une impulsion induite par un champ électrique en présence de Ca²⁺ 10 µM contenu dans une solution de glucose 0,3 M - 18 MΩ H₂O (milieu d'impulsion). Il est admis que, pendant que les pores sont ouverts, un flux d'ions circule selon les gradients de concentration à travers la membrane cellulaire vers l'intérieur du cytosol comme cela a déjà été montré dans des ovocytes d'oursin (Rossignol et al, 1983). Ainsi, l'influx ionique peut être ajusté dans ces conditions soit par la différence de concentration ionique entre l'intérieur et l'extérieur de la cellule soit par la durée des 25 impulsions.

La procédure expérimentale pour un flux ionique induit électriquement était similaire à celle précédemment décrite pour la fusion par champ électrique d'embryons de lapin à deux cellules (Ozil et Modlinski 1986). Les ovocytes sont cultivés avec du milieu M16 (Whittingham, 1971) à 38°C dans une chambre spécialement conçue. Avant chaque impulsion, le 30

milieu de culture est automatiquement remplacé par le milieu d'impulsion. Les détails de la chambre sont décrits dans la figure 1. Chaque impulsion était composée de deux impulsions alternatives afin d'éviter " une électrophorèse latérale " (Jaffe 1977) des protéines de membrane qui pourraient intervenir après plusieurs impulsions de la même polarité (Poo, 1981). L'amplitude du signal ionique était modulé à travers la durée de l'impulsion. Le processus en entier était contrôlé par un micro-ordinateur IBM PC-AT 286 par l'intermédiaire d'une interface Tektronix MI 5010 avec un programme écrit en MS-BASIC. Le voltage réel et le courant entre les électrodes étaient mesurés par un oscilloscope Tektronix 77041 monté avec un convertisseur numérique programmable 7D20 et un amplificateur 7A22.

Le rythme des impulsions électriques et la durée totale du traitement étaient les mêmes pour tous les traitements, c'est-à-dire appliquer une impulsion toutes les quatres minutes pendant 90 minutes. Ces valeurs ont été choisies parce qu'elles s'accordent bien avec la fréquence et la durée moyenne de l'hyperpolarisation du potentiel de membrane des ovocytes de lapin pendant la fécondation (22 oscillations biphasiques du potentiel membranaire pendant les premières 90 minutes suivant la fusion sperme-oeuf, une impulsion toutes les 4 minutes -McCulloh et al., 1983). On sait que la variation du potentiel de membrane reflète le passage de K basé sur des canaux activés par le calcium et qu'elles constituent donc un indicateur fiable de $[Ca^{2+}]_i$. (Miyazaki et Igusa 1982). L'amplitude de champs électriques (1.8 kVcm^{-1}) était constante pour tous les groupes expérimentaux.

25

Traitement des ovocytes

Les effets des différents paramètres du traitement sur l'activation des ovocytes ont été étudiés dans quatre groupes expérimentaux.

Groupe A - Environnement expérimental

5 Afin de tester l'effet des conditions expérimentales (perfusion continue, remplacement du milieu de culture et impulsions électriques), des ovocytes fraîchement ovulés et des oeufs fécondés soumis à 22 doubles impulsions de 900 µs dans le milieu d'impulsion dépourvu de Ca²⁺ (la première impulsion est donnée 13 à 15 heures après la saillie). Après le traitement, les oeufs fécondés ont été transférés dans des receveuses pour déterminer la survie à terme. Les ovocytes non fécondés ont été cultivés in 10 vitro et le taux d'activation parthénogénétique a été noté.

Groupe B - ions calcium et durée des impulsions

15 L'effet de la durée des impulsions a été étudié dans le milieu d'impulsion contenant du CaCl₂ 10 µM. Le traitement pour lequel les ovocytes ne sont pas activés a été considéré comme étant la durée minimum et celui résultant dans la lyse des ovocytes a été considéré comme étant la durée maximum. Un jeu de six traitements avec 22 doubles impulsions constantes a été choisi arbitrairement. Ces traitements ont une 20 durée d'impulsions égale à 200, 300, 600, 900, 1200 et 1500 µs respectivement. L'effet de la présence d'ions Na⁺ et Mg²⁺ à une concentration de 10 µM dans le milieu d'impulsion a été testé avec 22 doubles impulsions constantes d'une durée de 900 µs.

25 Groupe C - modulation des impulsions et type d'activation parthénogénétique

30 Le traitement avec 22 doubles impulsions de durée constante révèle la valeur de la durée de l'impulsion pour laquelle les effets maximum et minimum sont enregistrés. Ces traitements constants ne produisent pas un taux élevé d'activation avec un type uniforme d'oeufs parthénogénétiques. Afin de vérifier si une réduction progressive des

stimuli calcium dans un traitement donné a un effet sur le type de réaction parthénogénétique, les ovocytes ont été soumis à des traitements dans lesquels la durée des impulsions décroissait peu à peu selon une relation exponentielle négative. Quatre traitements ont été testés en accord avec la 5 durée maximum de la première impulsion. La figure 2 donne la courbe des durées d'impulsions pour ces traitements.

Une relation exponentielle négative ($D(n)=e^{(axn+b)+c}$) entre l'indice de l'impulsion (1 à 22) et la durée d'impulsion (1500 μs à 200 μs) a été choisie arbitrairement pour trouver la valeur de chaque impulsion au 10 cours du traitement.

Avec $D(n)$: durée d'impulsion du cycle (n).

Le coefficient a détermine la pente de décroissance de cette relation.

Le coefficient b détermine la durée de la première impulsion.

15 Le coefficient c détermine la durée de la dernière impulsion.

Les coefficients a et c sont constants et égaux respectivement à -0,4 et 200.

b = 5,9920 pour le traitement I
 = 6,5510 pour le traitement II
 20 = 6,9080 pour le traitement III
 = 7,1700 pour le traitement IV.

Groupe D - Modulation du champ électrique et développement in vitro

25 Les oeufs sont traités en présence de 8 $\mu g/ml$ -1 de cytochaliasine B dans le milieu de culture pour bloquer l'expulsion du second globule polaire et obtenir une population uniforme d'oeufs parthénogénétiques diploïdes. Deux traitements ont été appliqués au groupe C, le traitement I qui est le traitement faible avec une durée totale d'impulsion égale à 11 228 μs et le traitement III qui est le traitement fort avec une 30 durée de 14 868 μs . Les oeufs parthénogénétiques ont été cultivés in vitro jusqu'au stade blastocyste et l'influence des deux traitements a été évalué par le taux de formation de blastocyste.

Viabilité au delà de l'implantation des embryons parthénogénétiques

diploïdes

5 Les embryons au stade 4-cellules ont été transplantés dans les oviductes de receveuses. Les oeufs fécondés ont également été transplantés dans la corne opposée de quelques receveuses afin de comparer le développement parthénogénétique au développement normal. Les receveuses ont été autopsierées entre le jour 8.5 et le jour 13 de la gestation et le
10 nombre de sites d'implantation de foetus vivants a été noté.

RESULTATS

Groupe A - Effet de l'environnement expérimental (contrôles)

15 Quant le milieu d'impulsion ne contient pas d'électrolytes, aucun des ovocytes (105) n'est activé. L'environnement expérimental et les conditions de culture c'est-à-dire le remplacement du milieu de culture avant chaque impulsion par un milieu d'impulsion ne contenant pas d'électrolytes et le traitement par des impulsions électriques relativement fortes ($2 \times 1.8 \text{ kVcm}^{-1} \times 900\mu\text{s} \times 22$ fois, c'est-à-dire une durée totale d'impulsions de $30\ 600\ \mu\text{s}$) n'a pas d'effet visible sur les ovocytes fraîchement ovulés mais les conditions ne permettent pas de déclencher le développement. Par contre 41 % (9/22) des oeufs fécondés traités de la
20 même manière se sont développés à terme démontrant ainsi que le traitement par des impulsions électriques de haut voltage n'a pas d'effet contraire sur la capacité de développement des ovocytes fécondés. Il n'était pas possible de remplacer totalement le milieu de culture par le milieu d'impulsion avant chaque impulsion. Le courant mesuré pendant l'impulsion révèle que la conductivité du milieu d'impulsion est au moins de 15 % supérieure à celle du milieu d'impulsions mesurée entre les électrodes avant expérience et avant la première injection de milieu de culture.
25 Pendant l'expérimentation, des ions provenant du milieu de culture sont encore présents pendant les impulsions et ceci modifie le signal ionique. Ces variations du micro-environnement n'apparaissent pas comme ayant un quelconque effet significatif sur l'activation ou le développement embryonnaire. Dans cette série d'expérimentations, les oeufs sont lavés par
30

le milieu d'impulsion pendant 45 secondes toutes les quatre minutes. Cette période pendant laquelle les oeufs ne sont pas dans le milieu de culture n'a pas d'effet significatif sur l'activation ou sur la survie à terme.

5 Groupe B - ions calcium et durée de l'impulsion

Le tableau I résume les résultats de l'expérimentation dans lequel le taux d'activation a été testé en relation avec la durée d'impulsion en présence de CaCl_2 10 μM .

10 L'apparition de pronuclei après 3 ou 4 heures de culture sert de marqueur pour l'activation parthénogénétique. Ces résultats montrent clairement que l'activation parthénogénétique est déclenchée quand le milieu d'impulsion contient Ca^{2+} 10 μM .

15 De plus, le taux d'activation est directement relié à la durée de l'impulsion qui contrôle indirectement la stimulation par le calcium. La durée des stimuli ioniques n'influence pas seulement le taux d'activation mais aussi la configuration nucléaire des ovocytes parthénogénétiquement activés d'âge postovulatoire semblables.

20 Plus la durée d'impulsion est longue, plus la proportion d'oeufs activés est élevée mais plus la proportion d'ovocytes contenant des micronuclei est importante.

Groupe C - modulation des impulsions et type d'activation parthénogénétique.

25

Les résultats sont résumés dans le tableau II. La relation entre le type d'activation parthénogénétique et chaque traitement est également montrée dans le tableau III. Quand la durée de l'impulsion est réduite, tous les ovocytes sont activés et la majorité (91 %) d'entre eux ont un pronucleus unique et deux globules polaires quand la méiose est terminée.

30 Ainsi, la modulation des stimuli de calcium à travers la durée de l'impulsion semble être effective et influence les premières étapes du développement.

Dans cette étude, l'âge des ovocytes était similaire et ne peut donc pas affecter les résultats.

Groupe D - Effet de la modulation de champ électrique sur le

5 développement in vitro d'ovocytes activés par parthénogénèse.

Les résultats sont résumés dans le tableau III. Aucune différence visible dans le développement de chaque groupe n'a pu être notée jusqu'à la troisième division.

10 Dans les embryons parthénogénétiques produits par le traitement I, une proportion plus faible d'embryons présente une compaction et ceux qui se compactent sont irréguliers. Par contre, la majorité des embryons produits par le traitement III se compacte et se développe jusqu'au stade de blastocyste.

15 Ces résultats montrent que la forme du stimulus d'activation a un effet profond sur la capacité des embryons parthénogénétiques à se développer jusqu'au stade de blastocyste in vitro.

Viabilité post-implantation des oeufs parthénogénétiques diploïdes.

20

13 receveuses sont devenues gravides et ont été autopsiées entre le jour 8.5 et le jour 13. Les résultats sont résumés dans le tableau IV. Bien que les embryons parthénogénétiques soient plus petits que les embryons de contrôle, ils apparaissent morphologiquement normaux selon les critères définis pour le lapin par Edwards (1968). Le rapport entre la taille des annexes embryonnaires et celle du foetus est grossièrement équivalent pour les embryons obtenus par fécondation ou par parthénogénèse.

30 Ainsi, il semble que le développement général des embryons parthénogénétiques, le foetus et ses tissus trophoblastiques soit ralenti.

Les foetus morts ont plusieurs anomalies et il n'était pas possible de les classifier.

Tableau 1 -

Effet de la présence d'ions calcium et durée de l'impulsion électrique sur l'activation des ovocytes de lapin

5

| | Durée d'impulsion (μs) | Nombre d'ovocytes | Nombre lysés (%) | % activés | % d'ovocytes activés avec 1 PN+PB1 &PB2 | 2PN +PB1 | 2PN/# +PB1 |
|----|------------------------------|----------------------|---------------------|--------------|---|-------------|---------------|
| 10 | 200 μs | 47 | 0 (0) | 13 | 83 | 17 | 0 |
| | 300 μs | 99 | 0 (0) | 62 | 68 | 25 | 7 |
| | 600 μs | 85 | 0 (0) | 98 | 71 | 19 | 10 |
| | 900 μs | 63 | 0 (0) | 100 | 62 | 14 | 24 |
| | 1 200 μs | 97 | 5 (5) | 100 | 57 | 29 | 14 |
| 15 | 1 500 μs | 50 | 3 (6) | 100 | 30 | 17 | 53 |

Tableau 2 - Effet de la modulation du champ électrique sur l'activation
20

| | Traitement | Nombre d'ovocytes | % activation | % ovocytes activés avec 1 PN+PB1 &PB2 | 2PN +PB1 | 2PN/# +PB1 |
|----|------------|----------------------|-----------------|---|-------------|---------------|
| 25 | I | 86 | 88 | 83 | 9 | 8 |
| | II | 151 | 99 | 89 | 9 | 1 |
| | III | 118 | 100 | 91 | 9 | 0 |
| | IV | 96 | 100 | 70 | 25 | 5 |

30

Pronuclei anormaux, petits micronuclei

Tableau 3 -

Effet de la modulation de champ électrique sur le développement d'oeufs parthénogénétiques in vitro

| | <u>Traitement</u> | <u>Nombre</u> <u>d'ovocytes</u> | <u>%</u> <u>activation</u> | <u>Nombre</u> <u>cultivés</u> | <u>Nombre</u> <u>morulae</u> <u>compact (%)</u> | <u>Nombre</u> <u>blastocystes (%)</u> |
|--|-------------------|------------------------------------|-------------------------------|----------------------------------|---|--|
| | I | 98 | 91 | 69 | 25(36) | 23 (33) |
| | III | 352 | 100 | 244 | 244(100) | 216 (88) |

Tableau 4 -

Développement post-implantation d'oeufs parthénogénétiques soumis au traitement III.

| | <u>Jour de l'autopsie</u> | <u>J 8-9</u> | <u>J 9-10</u> | <u>J 10-11</u> | <u>J 12-13</u> | <u>Total</u> |
|----|---------------------------|--------------|---------------|----------------|----------------|--------------|
| | <u>Nombre d'oeufs</u> | | | | | |
| | <u>transférés</u> | 21 | 26 | 50 | 68 | 165 |
| | <u>Nombre d'implan-</u> | | | | | |
| | <u>tations</u> | 9 | 3 | 15 | 23 | 50 |
| | <u>% cumulé</u> | (42,8) | (21,2) | (27,8) | (30,3) | |
| | <u>Nombre de foetus</u> | | | | | |
| | <u>vivants</u> | 8 | 3 | 7 | 0 | 18 |
| | <u>% cumulé</u> | (88,8) | (91,6) | (66,6) | (36) | |
| 30 | | | | | | |

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REVENDICATIONS

1. Dispositif de culture de cellules constitué d'une enceinte comportant une arrivée de fluide et une sortie de fluide, caractérisée en ce qu'à la partie inférieure de l'enceinte, se trouvent un ou plusieurs orifices dont la géométrie est telle qu'elle s'oppose au passage des cellules et en ce que le fluide entrant dans l'enceinte est prélevé, au moins en partie, à travers les orifices, en créant une dépression telle qu'elle assure sensiblement le blocage des cellules sur le ou les orifices, une partie du fluide étant évacuée par débordement.
2. Dispositif selon la revendication 1, caractérisé en ce que des électrodes sont placées sur la paroi interne de l'enceinte.
3. Dispositif selon les revendications 1 et 2, caractérisé en ce que l'enceinte comporte deux parois latérales sur lesquelles sont placés les électrodes, le ou les orifices étant alignés à égale distance des électrodes.
4. Dispositif selon les revendications 1 à 3, caractérisé en ce que l'orifice est une orifice unique, constitué par une fente rectiligne dont la largeur est inférieure au diamètre des cellules.
5. Dispositif selon les revendications 1 à 4, caractérisé en ce que la fente est créée par la juxtaposition de deux plaques de verre.
6. Dispositif selon les revendications 1 à 5, caractérisé en ce que l'enceinte peut être isolée et placée dans une atmosphère de composition définie et/ou stérile.
7. Dispositif selon les revendications 1 à 6, caractérisé en ce que les arrivées et les sorties de fluide sont gérées par un système électronique.
8. Dispositif selon les revendications 2 à 7, caractérisé en ce que la fréquence, la durée et l'intensité des impulsions électriques arrivant dans les électrodes sont gérées par un système électronique.
9. Dispositif selon les revendications 1 à 8, caractérisé en ce que le fluide arrive dans l'enceinte par une tubulure courte, après passage par un système évitant l'arrivée de bulles de gaz de taille importante.

10 **10. Dispositif selon la revendication 9, caractérisé en ce que le système est constitué d'un ballon de verre dans lequel arrive le fluide et d'une tuyauterie d'évacuation qui est constituée d'un tube perforé de trous de très petit diamètre qui débouchent au voisinage du centre du ballon, 5 ladite tuyauterie se raccordant sur la tubulure courte.**

11 **11. Dispositif selon les revendications 1 à 10, caractérisé en ce que des fluides de compositions différentes peuvent arriver successivement dans l'enceinte, et que les systèmes d'arrivée et d'extraction des fluides évitent leur contamination réciproque.**

10 **12. Dispositif selon les revendications 2 à 11, caractérisé en ce que :**

- le fluide présent dans l'enceinte est un milieu d'impulsion, contenant au moins une substance assurant l'apparition d'un champ électrique et éventuellement une molécule ou toute substance spécifique que l'on veut faire pénétrer dans la cellule, et
- 15 - une impulsion de champ électrique est appliquée par l'intermédiaire des électrodes.

20 **13. Dispositif selon les revendications 1 à 12, caractérisé en ce que le système électrique permet de réaliser sur les cellules la séquence comprenant les étapes suivantes :**

- mise en culture des cellules dans un milieu de culture approprié pendant un temps déterminé,
- remplacement du milieu de culture par un milieu d'impulsion,
- 25 - envoi d'une impulsion de champ électrique par l'intermédiaire des électrodes,
- remplacement du milieu d'impulsion par un milieu de culture.

14. Dispositif selon les revendications 1 à 13, caractérisé en ce que les cellules traitées sont des ovocytes.

30 **15. Dispositif selon les revendications 1 à 13, caractérisé en ce que les cellules traitées sont des oeufs fécondés.**

16. Dispositif selon les revendications 1 à 15, caractérisé en ce que le milieu d'impulsion contient Ca^{2+} .

17. Dispositif selon les revendications 1 à 16, caractérisé en ce que le milieu d'impulsion contient Ca^{2+} à une concentration 10 μM .

5 18. Dispositif selon les revendications 1 à 17, caractérisé en ce que la séquence à laquelle sont soumises les cellules est répétée plusieurs fois, avec une fréquence pouvant varier, la durée de l'impulsion de champ électrique pouvant être différente au cours de chaque séquence.

19. Dispositif selon la revendication 18, caractérisé en ce qu'il permet de réaliser l'alignement des cellules et la fusion cellulaire par action d'une impulsion calibrée générée par un champ électrique.

10 20. Dispositif selon la revendication 19, caractérisé en ce que le traitement de fusion cellulaire est automatiquement géré par un système électronique.

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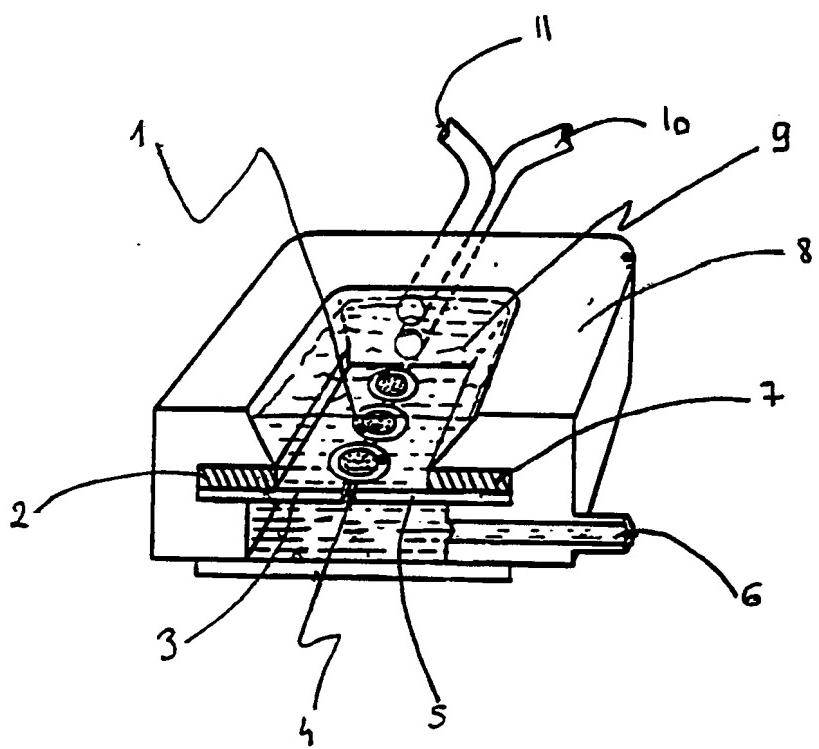


Figure 1

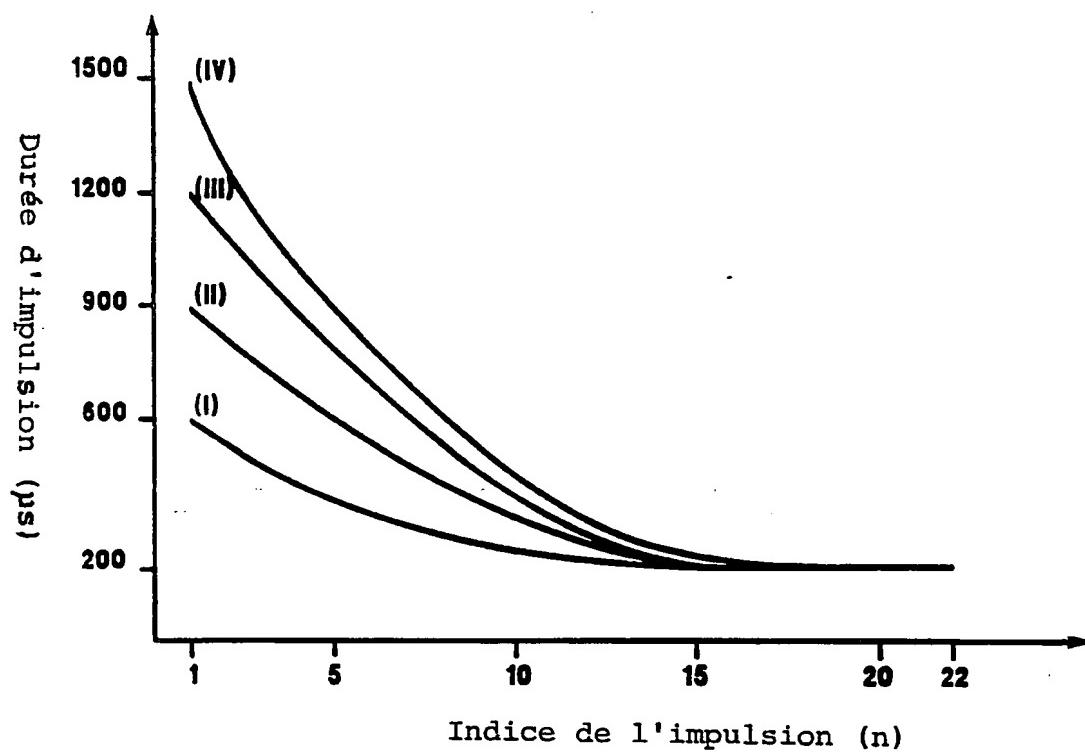


FIGURE 2

INSTITUT NATIONAL
de la
PROPRIETE INDUSTRIELLE

RAPPORT DE RECHERCHE

établi sur la base des dernières revendications
déposées avant le commencement de la recherche

FR 9003108
FA 442051

| DOCUMENTS CONSIDERES COMME PERTINENTS | | Revendications concernées de la demande examinée |
|--|---|--|
| Catégorie | Citation du document avec indication, en cas de besoin, des parties pertinentes | |
| X | US-A-4 894 343 (S. TANAKA et al.) * Fig.; revendications * --- | 1,4,5 |
| X | DE-A-3 829 028 (HITACHI LTD) * Fig. * --- | 1,4,5 |
| X | PATENT ABSTRACTS OF JAPAN, vol. 13, no. 487 (C-649)[3835], 6 novembre 1989; & JP-A-1 191 676 (HITACHI LTD) 01-08-1989 * Résumé * --- | 1-20 |
| X | PATENT ABSTRACTS OF JAPAN, vol. 13, no. 24 (C-561)[3372], 19 janvier 1989; & JP-A-63 230 070 (SHIMADZU CORP.) 26-09-1988 * Résumé * --- | 1-20 |
| X | PATENT ABSTRACTS OF JAPAN, vol. 13, no. 402 (C-633)[3750], 6 septembre 1989; & JP-A-1 144 968 (HITACHI LTD) 07-06-1989 * Résumé * --- | 1-20 |
| X | PATENT ABSTRACTS OF JAPAN, vol. 13, no. 84 (C-572)[3432], 27 février 1989; & JP-A-63 269 977 (HITACHI LTD) 08-11-1988 * Résumé * ----- | 1-20 |
| | | DOMAINES TECHNIQUES RECHERCHES (Int. CL5) |
| | | C 12 M C 12 N |
| Date d'achèvement de la recherche | | Examinateur |
| 14-12-1990 | | COUCKE A.O.M. |
| CATEGORIE DES DOCUMENTS CITES | | |
| X : particulièrement pertinent à lui seul Y : particulièrement pertinent en combinaison avec un autre document de la même catégorie A : pertinent à l'encontre d'au moins une revendication ou arrière-plan technologique général O : divulgation non-écrite P : document intercalaire | | |
| T : théorie ou principe à la base de l'invention E : document de brevet bénéficiant d'une date antérieure à la date de dépôt et qui n'a été publié qu'à cette date de dépôt ou qu'à une date postérieure. D : cité dans la demande L : cité pour d'autres raisons & : membre de la même famille, document correspondant | | |

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G01N 27/26

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G1N NBLE N25DX
U1S S1289 S1303

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On-line databases: WPI

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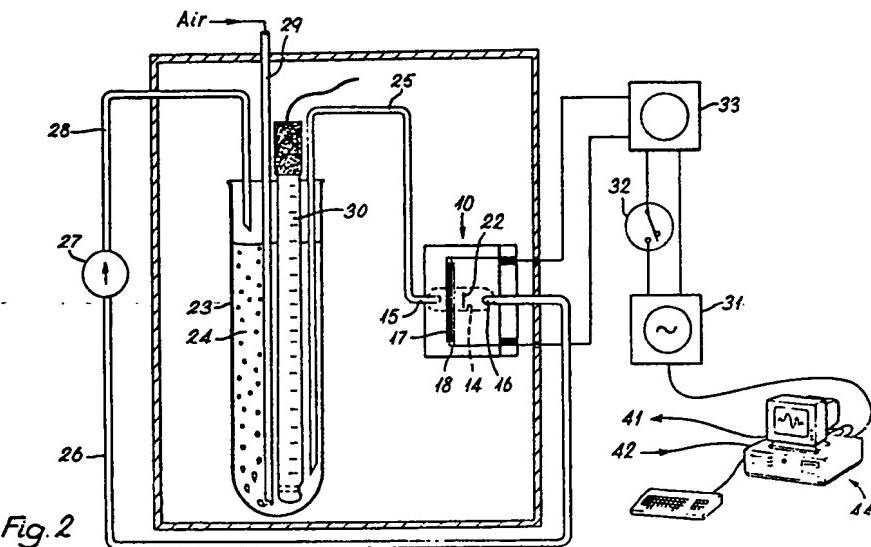
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(54) Dielectrophoretic characterisation of micro-organisms and other particles

(57) Dielectrophoretic collection rates of micro-organisms or other animate or inanimate dielectrically polarisable particles in suspension in a fluid are established by flowing the suspension past electrodes 17, 18 energised to produce a non-uniform alternating electric field in the suspension, terminating the energisation of the electrodes after a predetermined time to release particles collected from the suspension at the electrodes, and, downstream of the electrodes, measuring the pulse of released particles as a measure of the rate of particle collection during energisation of the electrodes. Repeated measurements at different field frequencies enable a collection-rate spectrum to be established and the particles under examination to be characterised or identified by reference to known spectra of known particles.

As described, a measuring cell 14 has inlet 15 and outlet 16 and electrodes 17, 18 arranged to produce a non-uniform field in the cell when supplied with AC from oscillator 31. The released particle concentration is determined at 22 by absorption of a light beam. The suspension is circulated by pump 27 and mixed in reservoir 23.



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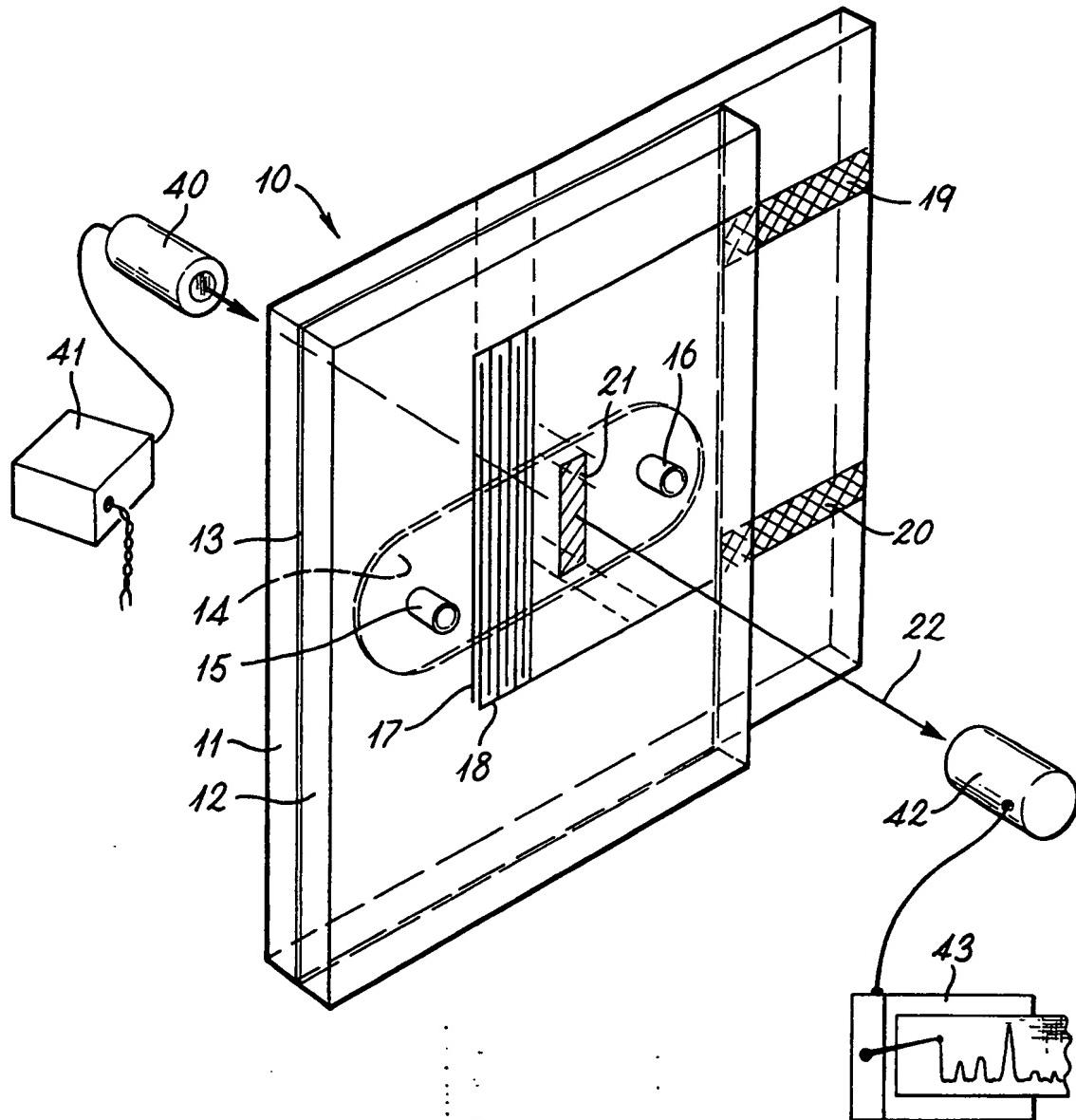


Fig. 1

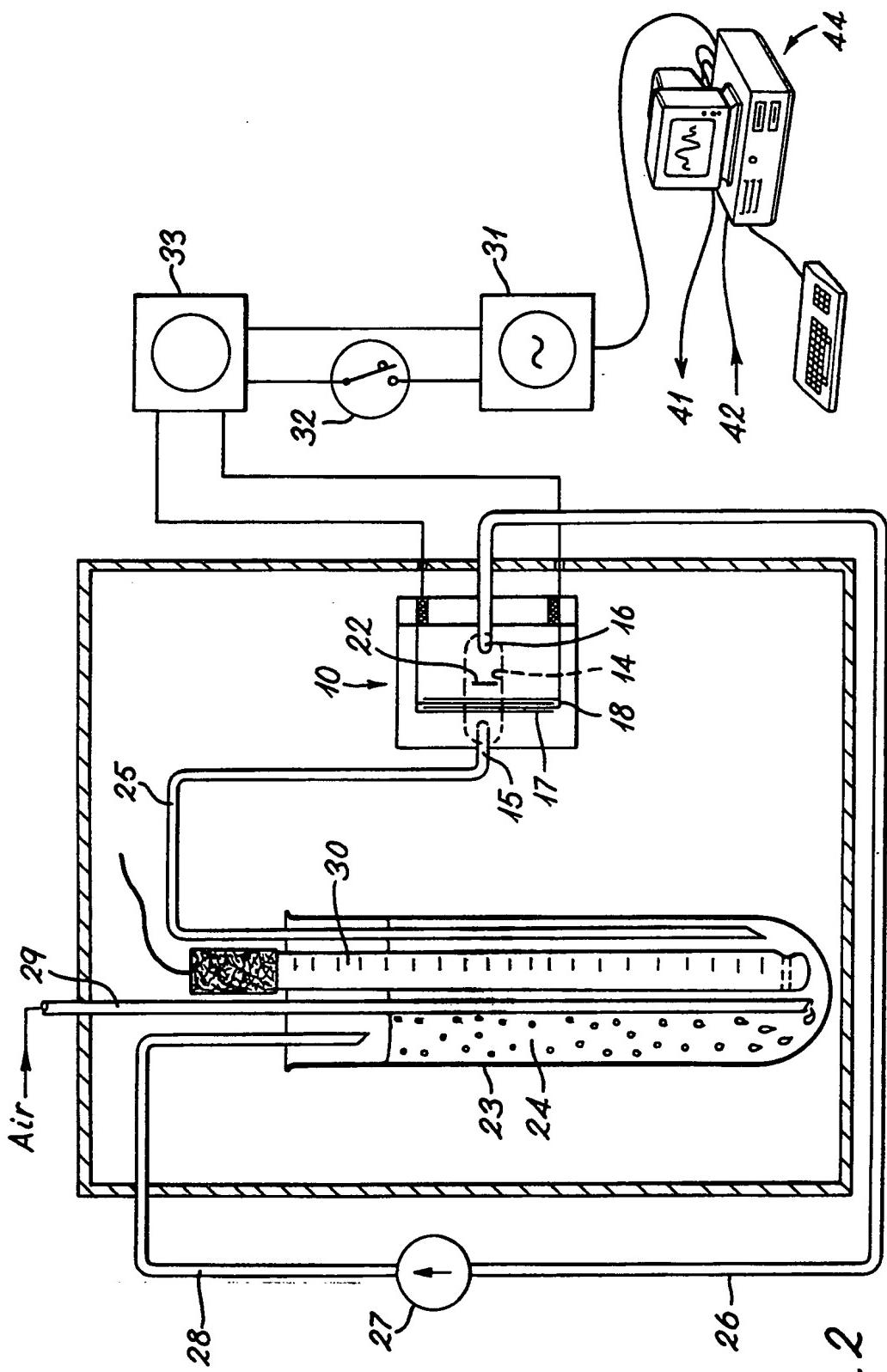


Fig. 2

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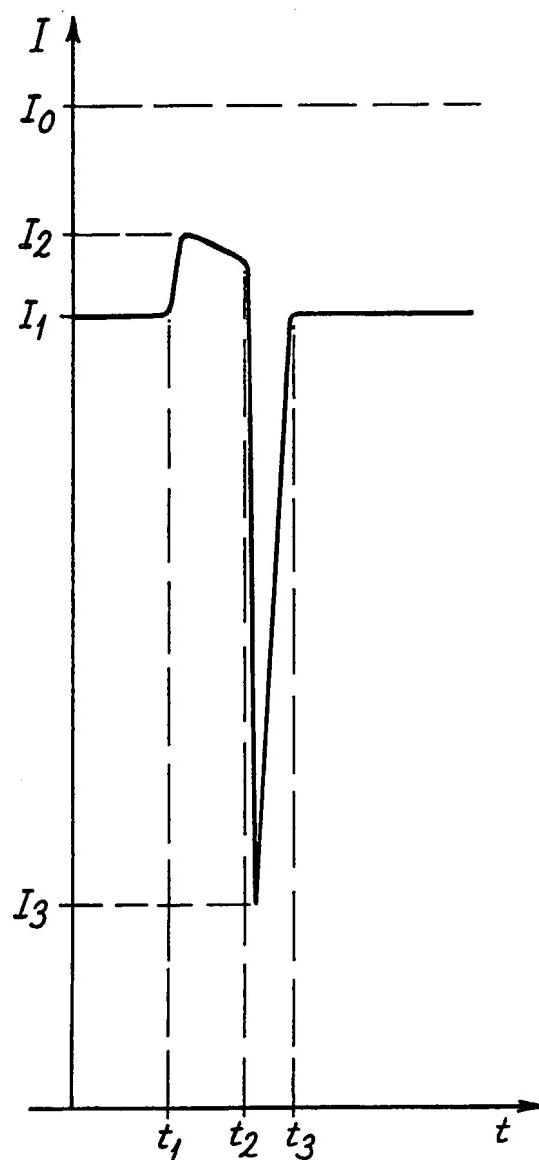


Fig. 3

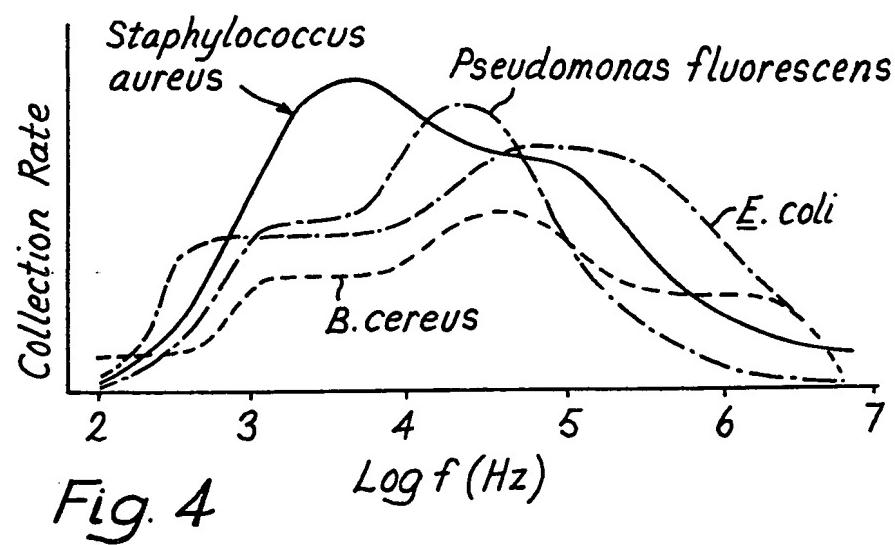


Fig. 4

- 1 -

DIELECTROPHORETIC CHARACTERISATION
OF MICRO-ORGANISMS AND OTHER PARTICLES

This invention relates to a method and apparatus for the characterisation or identification of micro-organisms and other particles, utilising the phenomenon of dielectrophoresis.

It is well known that dielectrically polarisable particles
05 suspended in a medium in a non-uniform electrical field are subject, even if they bear no net charge, to a "dielectrophoretic" force tending to move them (according as their polarisability is greater or less than that of the medium) in the direction of increasing or decreasing strength of the electric field, the force
10 F to which a particle of volume v and effective polarisability p is subject being given by the relation

$$F = pv (\mathbf{E} \cdot \nabla) \mathbf{E}$$

where E is the electric field strength at the position of the particle and ∇ is the del vector operator. In an alternating field
15 in which the field strength at any point is oscillatory but in which the field pattern remains stationary, the dielectrophoretic force on a particle is unidirectional, though its magnitude varies cyclically, and the resulting motion of the particle is also unidirectional, such as to move it, if its polarisability is
20 greater than that of the surrounding medium, towards increasing strength of field and, usually, towards one or another of a system of electrodes between which the field is produced. The use of an alternating field has the advantage that it imposes on a particle no net force due to any net electrical charge on the particle,
25 since any such force is itself alternating and its average over a cycle is zero.

It has been proposed to use the dielectrophoretic effect for collecting biological cells from an aqueous or other fluid suspension containing such cells, by placing the suspension in a
30 container provided with a system of electrodes so that the electrodes are immersed in the suspension, and then applying a voltage (usually alternating) between the electrodes so that cells in the suspension (moving always in the direction of increasing

field strength at their own immediate location) are caused to move towards one or other of the electrodes and to collect on the electrodes or in their immediate vicinity. As described in a paper by J.A.R. Price, J.P.H. Burt and R. Pethig in *Biochimica et Biophysica Acta* 964 (1988), pages 221-230, the rate of collection of the cells has been observed and measured photometrically by shining a light through the inter-electrode gaps and measuring the intensity of the beam of light after transmission; the reduction in transmitted light intensity, due to increased absorption or scattering of the light as cell collection proceeds, gives a measure both of the total of cells collected and of the rate of cell collection as a function of time. Usually the rate of collection is greatest initially and then falls off, due both to reducing concentration of cells remaining in the suspension and to a screening or saturation effect due to the presence at the electrodes of the cells already collected.

As reported in the above-mentioned paper, it has been found that the rate of collection of cells is also a function of the frequency of the applied electric field, i.e. of the voltage applied to the electrodes. For any one type of cell (or other particle) a collection-rate spectrum, i.e. a curve relating cell collection rate to frequency of the applied electric field, can be established over a field-frequency range from, say, 100 Hz to 10 MHz; and it is found that cells of different kinds have significantly different collection-rate spectra.

It might be hoped that this fact, that cells of different kinds possess different collection-rate spectra, might enable the unknown suspended constituents of a fluid suspension of micro-organisms to be identified by establishing a composite or aggregate collection-rate spectrum for the suspension as a whole and then analysing that spectrum in terms of the known spectra of individual possible constituents and the proportions in which such individual spectra could be combined additively to yield a composite spectrum corresponding to that established experimentally.

However, with the known apparatus by means of which such a composite spectrum could be established, the time and effort required for doing so would be inconveniently great since after every determination of the collection rate at one frequency the 05 container containing the sample of suspension being investigated would have to be flushed and filled with a fresh sample or, at least, the existing sample would have to be restored to its original pre-investigation condition, by vigorous agitation for example, in a manner which cannot easily be envisaged since it is 10 essentially stagnant. Furthermore, monitoring the collection rate by means of a light beam which shines only through inter-electrode gaps is an unsatisfactory expedient since it gives no direct information about collected particles which are concealed "behind" the electrodes.

15 It is an object of the present invention to provide improved apparatus for establishing dielectrophoretic collection rates and collection-rate spectra for dielectrically polarisable particles in a suspension and an improved method, using such apparatus, of establishing such collection rates and collection-rate spectra and 20 of thereby characterising or identifying such particles in suspension by reference to known collection-rate spectra of known types of particle. Particles to which the invention may be applied include various kinds of animate particles such as micro-organisms and cells such as blood cells, sub-cellular 25 particles such as viruses and plasmids, and inanimate-material particles such as latex beads, which may or may not be coated with animate materials; and in the following disclosure references simply to particles, for the sake of brevity, are intended to be understood in this broad way.

30 According to one aspect of the invention, there is provided apparatus for establishing dielectrophoretic collection rates for particles in a fluid suspension, comprising a chamber for the suspension fluid, an electrode system disposed to influence such fluid within the chamber, means for applying an alternating voltage between electrodes of the electrode structure and thereby 35

establishing in such fluid a spatially non-uniform alternating electric field thereby to induce dielectrophoretic collection adjacent such electrodes of electrically polarisable particles suspended in the fluid, and means for measuring particle
05 concentration at a location within the chamber, wherein the chamber is provided with an inlet and an outlet, so disposed that fluid flowing through the chamber from the inlet to the outlet flows past the electrode structure and then through the said location, and with means arranged to produce such fluid flow
10 through the chamber.

The means arranged to produce fluid flow through the chamber may be fluid circulating means arranged to re-circulate fluid from the outlet to the inlet of the chamber.

The means for measuring micro-organism or other particle
15 concentration is preferably of the kind already described, comprising a light source arranged to project a beam of light through the chamber (but at a location downstream of, instead of at, the electrode structure) and light detector means sensitive to the intensity of the light beam after transmission through the
20 chamber and thus to the increased or decreased absorption or scattering of the light beam which indicates an increase or decrease in the concentration of micro-organisms or other particles suspended in the fluid traversed by the light beam.

According to a further aspect of the invention there is
25 provided a method of establishing dielectrophoretic collection rates for dielectrically polarisable particles in a fluid suspension, comprising: causing the suspension fluid to flow past an electrode structure, energising the electrode structure for a predetermined time interval with an alternating voltage at a
30 preselected frequency and thereby establishing in the flowing fluid a spatially non-uniform alternating electrical field and inducing dielectrophoretic collection, adjacent the electrode structure, of dielectrically polarisable particles suspended in the fluid, thereafter terminating the energisation of the
35 electrode structure and thereby releasing the particles collected

adjacent thereto, and measuring the pulse of increased particle concentration at a location downstream of the electrode structure which occurs as the released particles are carried through such location by the flowing fluid.

05 Preferably this method according to the invention is carried out using the above-indicated apparatus according to the invention.

It will be appreciated that the pulse of increased particle concentration which is carried through the measuring location, 10 following release of collected particles from the electrode structure, is thereafter rapidly dispersed into the suspension fluid during its flow; and the method may be carried out repeatedly, even on a sample which is small and therefore has to be re-circulated, using different frequencies of the applied 15 alternating voltage during successive electrode-structure energisation intervals, in order to produce the data required for establishing either the whole or critical ranges of the collection-rate spectrum of the suspension under examination. The data thus acquired can then be correlated with corresponding known 20 data relating to the collection-rate spectra of particular micro-organisms or other particles to establish the relative and/or absolute concentrations of such particles required in the suspension under examination in order that it should yield the collection-rate data which have been acquired.

25 The invention will be more fully understood from the following more detailed description with reference to the accompanying drawings, in which:-

Figure 1 is a diagrammatic perspective view of a chamber assembly provided with an electrode system for use in accordance 30 with the invention;

Figure 2 is a diagrammatic representation of apparatus according to the invention, incorporating the chamber assembly shown in Figure 1;

Figure 3 is a representation of the manner in which absorption 35 of a light beam provided in the apparatus of Figure 2 varies with

time during and after a micro-organism or other particle collection period while the apparatus is in use; and

Figure 4 is a representation of collection-rate spectra of four different micro-organisms, as established by previous workers
05 and available in the published prior art.

The chamber assembly shown in Figure 1 and indicated generally by the reference 10 comprises a back plate 11 and a front plate 12, both made of glass, and transparent, with a spacer sheet 13 sandwiched between them. A central part of the spacer 13 is
10 removed so as to form a thin chamber 14 (the thickness of the spacer 13, which may be about 0.05 mm but may be within a wide range, depending on the sizes of suspended particles which may be encountered) between the plates 11 and 12, and the plate 12 is provided with an inlet 15 and an outlet 16 open to the chamber
15 14. The chamber 14 may be some 10 mm in height and 40 mm in length. The back plate 11 has upon it an electrode structure in the form of a metallic layer, for example of aluminium, deposited upon it to a thickness of, say, 1 micron and then etched to provide a pair of interdigitated electrodes 17 and 18 integral
20 with connection terminal tabs 19 and 20 respectively. Each electrode may be formed with eight parallel fingers each 0.06 mm in width and separated by 0.06 mm from each adjacent finger of the other electrode, and the central part of the length of each finger is exposed to the interior of the chamber 14 to be in close
25 proximity to a fluid disposed therein, though a protective film of insulating material may be provided to prevent actual contact between the fluid and the electrodes. The shape of the electrodes is such as to provide a spatially very non-uniform electrical field in their immediate vicinity when a voltage is applied
30 between them. The electrodes 17 and 18 are nearer to the inlet 15 than to the outlet 16, leaving between the electrodes and the outlet 16 a region 21 of the chamber 14 through which a beam of light (for example of 450 or 660 nm wavelength, or another wavelength more suitable for a particular material) indicated by
35 an arrow 22 may be arranged to shine without being obstructed by

the electrodes 17 and 18.

The chamber assembly 10 shown in Figure 1 is incorporated in apparatus according to the invention shown in Figure 2. This comprises a reservoir 23 of a liquid suspension 24 containing particles, say micro-organisms, which are to be identified. A tube 25 connected to the inlet 15 of the chamber assembly 10 dips into the liquid suspension 24 in the reservoir 23, and the outlet 16 of the chamber assembly is connected by a tube 26 to a pump 27, which may be a peristaltic pump, which draws suspension fluid through the chamber 14 and returns it to the reservoir 23 via a return tube 28, for example at a rate between 0.1 and 1.0 ml per minute. Air from an airline 29 bubbles through the suspension 24 in the reservoir and serves both to agitate the suspension and to keep it aerated. Also extending into the reservoir 23 is a pH probe 30 for monitoring the pH of the suspension 24 to enable it to be maintained at a desired constant level, since it is found that the collection rate of micro-organisms by dielectrophoresis at the electrodes 17 and 18 is dependent on the pH of the suspension. Preferably the whole apparatus is maintained at a desired constant temperature, since temperature variation also tends to affect collection rates.

The apparatus also comprises a signal generator 31 producing an alternating voltage at selected frequency and amplitude which may be applied, by means of a switch 32, to an oscilloscope 33, which serves to monitor it, and to the electrodes 17 and 18 of the chamber assembly 10. Conveniently, the voltage applied to the electrodes has an amplitude selected in the range between 5 and 30 volts, and frequencies which range from 10 Hz to 10 MHz or more. Also provided are a light source, preferably an LED light source 40 energised by a power supply 41 as shown in Figure 1, arranged to project the light beam 22 through the chamber 14, and a photometer shown as 42 in Figure 1 which measures the intensity of the beam 22 after it has passed through the chamber 14 and provides an input for a chart recorder 43.

In use of the apparatus, with the pump 27 continuously drawing

a flow of the suspension 24 through the chamber 14 and re-circulating it to the reservoir 23, the switch 32 is closed for a period of, say, 5 seconds to apply the voltage from the signal generator 31, at a predetermined amplitude and selected frequency,
05 to the electrodes 17 and 18 and produce a spatially non-uniform alternating electric field in the suspension adjacent the electrodes, resulting in micro-organisms in the suspension being moved dielectrophoretically and collected on or adjacent the electrodes. When the switch 32 is opened, the collected
10 micro-organisms are released and carried downstream by the continuing flow of the suspension liquid, to pass through the light beam 22 as a localised pulse of increased concentration of micro-organisms in the suspension.

The resulting form of an output signal from the photometer,
15 recorded by the chart recorder 43 and representing the measured intensity I of the light beam 22 as a function of time t , is indicated in Figure 3. In the absence of an applied voltage on the electrodes 17 and 18, a steady measured beam intensity I_1 is less than the value I_0 which would represent the beam intensity
20 before it passes through the chamber assembly 10. The difference $I_0 - I_1$ represents the intensity loss during passage through the chamber assembly 10, due largely to absorption and/or scattering of light by micro-organisms suspended in the liquid flowing through the chamber 14. When an alternating voltage is applied to
25 the electrodes 17 and 18 at time t_1 , the measured light intensity rises sharply to a value I_2 as micro-organisms begin to be collected on or adjacent the electrodes and their concentration in the fluid downstream, as it passes through the light beam 22, is rapidly reduced. In the interval until time t_2 when the applied
30 voltage is switched off, the collection rate at the electrodes begins to fall off and the measured intensity of the light beam begins to fall as the micro-organism concentration downstream of the electrodes begins to rise correspondingly. Removal of the applied voltage at time t_2 results in a sudden release from the
35 electrodes of the collected micro-organisms which are carried

downstream as a highly localised pulse of increased micro-organism concentration in the flowing suspension, resulting in a sharp reduction in the beam intensity to a low value I_3 as the pulse passes through the beam. At a slightly later time t_3 , the pulse
05 has passed and the measured beam intensity has returned to its steady value I_1 . The increase in absorption represented by the intensity difference I_1-I_3 is (by a factor of perhaps 100) a much more sensitive measure of the quantity of micro-organism(s) collected in the interval from t_1 to t_2 , and thus of the initial
10 collection rate, than is the relatively small rise in intensity from I_1 to I_2 which is the direct consequence of the collection rate.

Once the pulse of increased-concentration suspension has passed through the light beam 22, it is rapidly dispersed as it is pumped back into the reservoir 23 and is there further agitated by air from the air line 24. Successive applications of alternating voltage to the electrodes 17 and 18 at different frequencies, preferably automatically, under the control of a computer 44 as shown schematically in Figure 2, can follow one another in quick succession to establish the data which, stored by the computer, will define the collection-rate spectrum of the fluid under examination. Thus the time required to obtain a collection spectrum over a frequency range from 10 Hz to 1 MHz may be reduced from more than a day, by methods known hitherto, to 5 minutes or
20 less. Comparison of the defining data of the spectrum thus obtained with corresponding data from known spectra of selected individual micro-organisms or other possibly relevant particles, to obtain an analysis of the particle content of the sample under examination, can also be rapidly effected using a suitable computer program, so that analyses of samples can be rapidly performed using the apparatus and method of the invention.
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Collection-rate spectra for four micro-organisms as known from previous work are shown in Figure 4, in which the curves 34, 35, 36 and 37 respectively represent the collection-rate spectra of
35 Staphylococcus aureus, Pseudomonas fluorescens, E. coli and

B. cereus. Rather than adopt such previous results uncritically as reference spectra, it may be preferable for use with a particular apparatus according to the invention to build up a library of such reference spectra as obtained using that apparatus
05 with the operating conditions established as they will be set during subsequent use of the apparatus. In general, however, the apparatus requires only a very low level of routine calibration, while nevertheless providing a markedly increased sensitivity,
10 greater selectivity for microbial and other particle types and much improved speed and simplicity of operation as compared with previously available apparatus.

As mentioned above, the electrodes 17 and 18 may be of aluminium, and formed by depositing a layer of the metal on the glass plate 11 and then etching to provide the required electrode pattern. Instead of aluminium, platinum or gold-plated chromium
15 electrodes may be employed, produced either by an etching technique or by a "lift-off" technique in which a pattern mask is formed on the substrate, using a suitable material such as a photoresist material, before a metal layer is deposited and
20 unwanted regions of deposited metal are then removed by removing the pattern mask so as to leave the metal only where it was deposited directly on the substrate.

CLAIMS

1. Apparatus for establishing dielectrophoretic collection rates for particles in a fluid suspension, comprising a chamber for the suspension fluid, an electrode system disposed to influence such fluid within the chamber, means for applying an alternating voltage between electrodes of the electrode structure and thereby establishing in such fluid a spatially non-uniform alternating electric field thereby to induce dielectrophoretic collection adjacent such electrodes of electrically polarisable particles suspended in the fluid, and means for measuring particle concentration at a location within the chamber, wherein the chamber is provided with an inlet and an outlet so disposed that fluid flowing through the chamber from the inlet to the outlet flows past the electrode structure and then through the said location, and with means arranged to produce such fluid flow through the chamber.
2. Apparatus as claimed in Claim 1, wherein the means arranged to produce fluid flow through the chamber is fluid circulating means arranged to re-circulate fluid from the outlet to the inlet of the chamber.
3. Apparatus as claimed in Claim 1 or Claim 2, wherein the means for measuring particle concentration comprises a light source arranged to project a beam of light through the chamber at the said location, downstream of the electrode structure, and light detector means sensitive to the intensity of the light beam after transmission through the chamber and thus to the increased or decreased absorption of scattering of the light beam which indicates an increase or decrease in the concentration of particles suspended in the fluid traversed by the light beam at the said location.
4. Apparatus for establishing dielectrophoretic collection rates for particles in a fluid suspension, substantially as described herein with reference to the accompanying drawings.

5. A method of establishing dielectrophoretic collection rates for dielectrically polarisable particles in a fluid suspension, comprising: causing the suspension fluid to flow past an electrode structure, energising the electrode structure for a predetermined time interval with an alternating voltage at a preselected frequency and thereby establishing in the flowing fluid a spatially non-uniform alternating electrical field and inducing dielectrophoretic collection, adjacent the electrode structure, of dielectrically polarisable particles suspended in the fluid, thereafter terminating the energisation of the electrode structure and thereby releasing the particles collected adjacent thereto, and measuring the pulse of increased particle concentration at a location downstream of the electrode structure which occurs as the released particles are carried through such location by the flowing fluid.
6. A method of establishing a dielectrophoretic collection-rate spectrum for particles in a fluid suspension, comprising carrying out the method of Claim 5 repeatedly, using different frequencies for the applied alternating voltage during successive electrode-structure energisation intervals, thereby to produce the data required for establishing either the whole or critical ranges of the collection-rate spectrum of the suspension.
7. A method of identifying dielectrically polarisable particles contained in a fluid suspension, comprising establishing dielectrophoretic collection-rate spectrum data therefor by the method claimed in Claim 6 and correlating the data thus acquired with corresponding known data relating to the collection-rate spectra of particular identified particles, thereby to establish the relative and/or absolute concentrations of such identified particles required to be present in the suspension under examination in order that it should yield the collection-rate data which have been acquired.
8. A method as claimed in any one of Claims 5, 6 and 7, carried out by means of apparatus as claimed in any of Claims 1 to 4.

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(54) Sensor

(57) The sensor is for detecting a substance and includes a biological component in which electron transport may be influenced by the presence of the substance and optical means for detecting a parameter related to electron transport in the biological component or part thereof.

The invention also provides a method for detecting a substance and a process for preparing a sensor.

The sensor finds one application, by way of example, in monitoring the potability of water.

GB 2 239 311

1.

Sensor

The present invention relates to a sensor, a method of detecting and a process for preparing a sensor.

According to one aspect of the present invention there is provided a sensor for detecting a substance which sensor includes a biological component, in which electron transport may be influenced by presence of substance to be detected, and optical means for detecting a parameter related to electron transport in the biological component or a part thereof.

According to another aspect of the present invention there is provided a method for detecting a substance which method includes the steps of subjecting a biological component, in which electron transport may be influenced by presence of a substance to be detected, to the influence of the substance and detecting by optical means, which means includes an emitter and a detector, a parameter related to electron transport in the biological component or part thereof.

The biological component may be, for example, any suitable biological component capable of providing an electron transport chain.

One type of electron transport chain, namely a photosynthetic electron transport chain, is mentioned in the publication "Biosensors". 4 (1989) pages 299-311 (Elsevier Science Publishers Limited, England); the present invention may utilise such a chain but, as hereinafter disclosed, is not limited to photosynthetically driven electron transport chains.

It will be appreciated that an electron transport chain may also be referred to as an electron transfer chain.

The biological component may be, for example, a membrane within a cell (e.g. a thylakoid membrane) or part of the cell membrane of a cell; where, for example, the biological component is provided by using a whole cell, any suitable cell may be used to provide a biological component comprising, for example, a membrane having an electron transport chain.

Examples of types of cell which may be used as or to provide a biological component are bacteria (e.g. a cyanobacteria microorganism such as *Synechococcus*), algae (e.g. green algae), yeasts and plant cells.

It is to be understood that, for example, any suitable biological component capable of providing an electron transport chain may be utilised in accordance with the present invention. Thus, for example, a photosynthetically driven system may be used (i.e. a system having a photosynthetically driven electron transport chain may be used).

However, the invention is not limited to the use of such systems nor to systems which utilise electrons obtained from the splitting of water.

Thus, for example, if desired, other systems such as those driven by other energy sources may be used (e.g. a biological component or system may be used in which the catalysis of organic molecules such as carbohydrates (e.g. glucose) provides electrons to be transported along the electron transport chain). By way of further example, a mitochondrial electron transport chain may be utilised.

It is also to be understood that, in accordance with the present invention, the biological component may comprise, for example, a single component of an electron transport chain (e.g. a protein molecule) rather than a complete electron transport chain; where a single component of an electron transport chain is used such a component may be, for example, immobilised. Any suitable immobilisation technique may be used (e.g. adsorption, covalent

attachment, micro-encapsulation or physical entrapment).

The substance to be detected may be, for example, any substance which is capable of influencing electron transport in the biological component such as to permit a parameter related to electron transport in the biological component or part thereof to be detected by optical means.

Thus, for example, where the sensor is to be used to indicate the potability of water, the substance may be a toxic substance or a pollutant such as one of those appearing at page 1 of United Kingdom Patent Application No. 2189605A. However, it will be appreciated that the present invention is not limited to detecting toxic substances or pollutants; thus, the invention may be utilised in the detection of, for example, other substances the presence of which it is desired to detect (e.g. chemical species which may not necessarily be regarded as a toxic substance or a pollutant).

The term "substance", meaning the substance to be detected, in this specification, embraces a substance as such or a precursor therefor.

It will be understood that the substance to be detected may influence electron transport in an electron transport chain by, for example, interfering with electron transport in the electron transport chain such as to prevent electron transport within the chain.

Also, it is to be understood that the substance to be detected may influence electron transport in an electron transport chain by, for example, interfering with a mechanism by which electrons are made available for transport along the chain.

Thus, for example, where an enzymatic reaction is used to produce electrons for subsequent transport along the electron transport chain, any substance which interferes with the enzymatic reaction such as to affect the availability of electrons for subsequent transport along the chain may be detected by detecting by optical means a parameter related to electron transport along the electron transport chain (e.g. electron flux in the electron transport chain may be utilised to detect presence of a substance which interferes with the enzymatic reaction).

Further, it is to be understood that electron transport in the electron transport chain may be monitored for example by detecting the presence or absence of an electron in the biological component or part thereof, or by detecting the frequency of presence or absence of an electron in the biological component or part thereof; this monitoring may be considered to be monitoring the state of electronic transport in the electron transport chain.

Thus, for example, where the electron transport chain includes a collection of protein molecules which chain at one end gives rise to a change in mediator oxidation/reduction state (e.g. NADPH oxidation/reduction state) due to electron transport through the chain following a suitable event at the other end of the chain (e.g. following the splitting of water or other suitable mechanism giving rise to an electron for transport through an electron transport chain), any substance which inhibits transport of electrons in the chain will affect the state of electron transport in the chain and therefore will affect the oxidation/reduction state of the mediator.

It has previously been proposed in the art to use an artificial mediator in conjunction with a whole cell as a basis for a sensing device; reference may be made in this context to the pages of the publication "Biosensors" 4 (1989) hereinbefore mentioned.

In such a sensing device a natural mediator (e.g. NADPH) is replaced by an artificial mediator (e.g. potassium ferricyanide) which can be oxidised at an electrode to give an electrical signal. It will be understood that any substance which inhibits electron transport in the electron transport chain may result in a reduction in the amount of artificial mediator available in a suitable state for oxidation at the electrode with a resulting reduction in electrical signal.

However, a sensor device such as that to which reference was made immediately hereinbefore may suffer from one or more disadvantages such as the following:

- (a) continuous addition of an artificial mediator may be required;
- (b) since the artificial mediator tends to starve a cell of energy, continuous exposure of the cell to

artificial mediator tends to limit sensor lifetime, thus necessitating replacement after a relatively short time;

(c) transport of electrons from the cell to the electrode is generally the rate determining step;

(d) the sensing device may be relatively non-specific since anything which interferes with the electron transport chain may give rise to a reduction in electrical signal generation at the electrode.

By way of further explanation in relation to (d) of the immediately preceding paragraph it may be noted that electron transport may be inhibited at any one of a number of points on the chain depending upon the substance present to cause interference. Thus, for example, where an electron transport chain comprises a collection of protein molecules, such as those present in the cell membrane of a cell such as a cyanobacteria, electron transport between any two molecules in the chain may, in principle, be interrupted. Thus, although one substance may serve to inhibit electron transport at, say, one point "up stream" of the chain access point for benzoquinone (thus influencing mediator production) another substance may inhibit electron transport at another point in the chain such as to influence the oxidation/reduction state of the mediator.

Accordingly, the sensing device of the prior art may detect the presence of interfering substances without the ability to distinguish between said substances.

The sensor of the present invention may be used substantially to avoid or overcome one or more of the disadvantages hereinbefore disclosed.

Thus, in accordance with the present invention no artificial mediator as hereinbefore mentioned is required since it is a parameter related to electron transport in the biological component or part thereof which is utilised.

Furthermore, the optical means for detecting the parameter of the biological component and/or the biological component may be

chosen, for example, such that the parameter is characteristic of the biological component or part thereof so as to offer the possibility of greater specificity in the detection of substances.

Thus, for example, in one embodiment of the present invention, the parameter to be detected may be an absorption spectrum, or part thereof, of the biological species or part thereof.

The absorption spectrum may be, for example, in the visible/u.v. wavelength regions. However, the present invention is not limited to such wavelength regions and thus as used in the Specification "optical means" is not limited to any particular wavelength.

Furthermore, the optical means is not, by way of further example, limited to those for detecting absorption spectra since any suitable optical means for detecting a parameter related to electron transport in the biological component, or part thereof, may be used in accordance with the present invention.

Thus, by way of further example, an entity may be included in the sensor which entity is capable of emitting energy upon receiving an electron from the biological component or part thereof thereby to enable emission from the entity to be detected by optical means to indicate the presence or absence of a substance to be detected; such an entity may be, for example, a fluorophore the light emission of which may be triggered by electron acceptance.

It will be appreciated that the presence of a substance to be detected may influence electron transport in the biological component or part thereof (e.g. a component or components of an electron transport chain) such as to give rise to an electron or electrons for reception by the entity.

Thus, for example, a substance to be detected may inhibit electron transport along an electron transport chain such that an electron becomes or electrons become available to interact with the entity at an intermediate position along the electron transport chain.

By way of example, the entity may be substantially non-toxic with respect to the biological component or part thereof.

By way of further example, an entity may be included in the sensor which entity comprises a fluorescent redox-active species

(which may also be considered to be a fluorescent redox-active mediator) which species is capable of accepting an electron from the biological component or part thereof and capable of exhibiting fluorescence (e.g. emission or excitation) in one of its redox states (e.g. fluorescent when in the oxidised form and non-fluorescent when in the reduced form). Also, by way of example, the biological component or part thereof may be a component or components of an electron transport chain (e.g. such as that of a photosynthetic electron transport chain or of a mitochondrial electron transport chain).

Where a fluorescent redox-active species is used, the presence or absence of a substance to be detected may be determined, for example, by observing the oxidation state of the fluorescent redox-active species as indicated by its fluorescence characteristics or lack of fluorescence.

It will be understood that by selecting the fluorescent redox-active species it may be possible to arrange for fluorescence to occur either in the presence of a substance to be detected or in the absence of a substance to be detected.

It will also be understood that, by way of example, an excitation energy may be applied to the fluorescent redox-active species to cause it to produce fluorescent emission. By way of example the wavelength of the excitation energy and the wavelength of the fluorescent emission may be arranged so as not to coincide with wavelengths which give rise to substantial absorption by or emission from materials other than those involved in the detection of the substance to be detected; in this way interference from, for example, natural biomolecules may be substantially avoided.

The fluorescent redox-active species may be selected, for example, to have a redox potential which is at least 200mV more positive than the biological component or part thereof so as to encourage electron transfer.

The fluorescent redox-active species may have, for example, a molecular structure which facilitates the formation of a charge transfer complex to promote electron transport.

By way of further example the fluorescent redox-active

species may be incorporated into an immobilised microorganism layer said layer providing the biological component or part thereof in which electron transport may be influenced by the presence of the substance to be detected.

Such incorporation may be achieved, for example, by non-specific doping into a growth medium or by specific covalent attachment (e.g. to a cellular or electron transport component).

The choice of fluorescent redox-active species may be used, for example, to select the substance which may be detected; thus, for example, points on the biological component or part thereof (e.g. points on an electron transport chain) which cannot interact with species due to energetic (redox) reasons or steric reasons (e.g. the inability to form an effective charge-transfer complex) will not lead to a change in the oxidation state of the species.

Thus, the present invention further provides a method for detecting a substance which method includes the steps of subjecting a biological component, in which biological component or part thereof electron transport may be influenced by the presence of a substance, to the influence of the substance, said biological component being in communication with a fluorescent redox-active species capable of accepting an electron from the biological component or part thereof and said species being capable of exhibiting fluorescence in one of its redox states, and observing the fluorescence characteristics of the said species.

By way of further example, the present invention provides a sensor which includes a biological component which biological component or part thereof is such that it is capable of giving rise to fluorescence (e.g. by emission and/or excitation) the fluorescence transient characteristics of which may be affected by the presence of a substance which influences electron transport in the biological component or part thereof, and means for detecting the fluorescence transient characteristics.

It will be understood, therefore, that detecting by optical means a parameter related to electron transport in a biological component or part thereof, embraces detecting by measurement and/or observation of kinetic or transient characteristics of an optical

phenomenon (e.g. fluorescence kinetics, or kinetic or transient responses of an optical phenomenon); the kinetic or transient characteristics may be, for example, those of any suitable biological component or part thereof (e.g. an electron transport chain or a specific component of an electron transport chain).

The biological component may be, for example, immobilised. Any suitable immobilisation technique may be used (e.g. adsorption, covalent attachment, micro-encapsulation or physical entrapment).

The application of photo-energy to a suitable biological component (e.g. illumination by light of a suitable wavelength) may produce, for example, fluorescence which has two distinct fluorescence components namely an immediate fast fluorescence transient component and a subsequent slow ("delayed") fluorescence transient component.

The presence of a substance which affects electron transport in the biological component, or part thereof, such as to influence either or both of these fluorescence components may be detected by observing the fast transient component and/or the slow transient component.

For example, where a biological component or part thereof is such that electron transport therein involves the transport of electrons into a photosystem II function, the presence of a substance which inhibits such electron transport may affect the fluorescence transient components and thus be detected (e.g. the substance may cause an increase in the fast fluorescence transient component and substantially reduce or abolish the slow fluorescence transient component).

Examples of biological components which may have the ability to exhibit fast and slow fluorescence transient components, which components may be influenced by the presence of a substance to be detected, are algae, photosynthetic bacteria and chloroplasts, which contain photosynthetic reaction centres having photopigment complexes.

For example, phycobiliproteins possess fluorescence bands in the range 580-680nm and various forms of chlorophyll fluoresce in the 680-750nm region. Selection of a suitable excitation wavelength may enable, for example, measurement of fluorescence emissions due to chlorophyll only (e.g. the Chla band at 685nm).

A sensor in accordance with the present invention therefore may, for example, include an immobilised layer of a photosynthetic bacterium or alga in communication with an optical transduction system.

The optical transduction system may include for example, a means for effecting the controlled excitation of fluorescence in the bacterium or alga and/or means for detection and/or measurement of energy emitted in response.

In accordance with an embodiment of the present invention the fast fluorescence transient component may be observed at a predetermined time or times so as to permit the presence or absence of a substance to be detected; the fast fluorescence transient component may, for example, be observed at a predetermined time or times prior to fluorescence saturation.

In one example of a sensor in accordance with the present invention photosynthetic bacterial cells or alga cells may be maintained in a substantially dark environment and be subjected to pulses of light of a selected wavelength (e.g. 600nm) and selected duration (e.g. 1 minute illumination followed by a 1 minute period of darkness) and the intensity of fluorescence may be measured at a predetermined time interval (e.g. 0.5 sec.) after the onset of illumination in each pulse.

It will be appreciated that the presence of a substance (e.g. a photosystem II inhibitor) which influences electron transport in the cells or part thereof may be detected by comparing the characteristics of fluorescence transient components with the characteristics of fluorescence transient components in the absence of the substance.

It will also be appreciated that, by way of example, the pulses of light may serve the dual purposes of maintaining cell viability by stimulating photosynthetic energy generation and inducing detectable fluorescence (e.g. in the 685nm band).

In one embodiment of the first aspect of the present invention hereinbefore disclosed there is provided a sensor for detecting a substance which sensor comprises a biological component and optical means for detecting a parameter related to electron

transport in the biological component or part thereof wherein the optical means for detecting a parameter related to electron transport in the biological component or part thereof includes an emitter and a detector.

The emitter may be any suitable emitter means, examples of which are a laser, a light emitting diode and an optical fibre.

The detector may be any suitable detector means such as a photo-detector, for example.

The emitter and detector may be, for example, operated in accordance with suitable known techniques using suitable known apparatus as required.

In another embodiment of the present invention there is provided a sensor for detecting a substance which sensor comprises a layer of cells, capable of providing a biological component, sandwiched between an optical emitter and an optical detector.

Where, by way of example, an emitter and a detector are used in accordance with the present invention the emitter and detector may be arranged, for example, to co-operate so that together they form an optical means which may be specific to one wavelength or to a number of wavelengths.

Thus, for example, an optical means comprising an emitter and a detector may be arranged to be specific to one wavelength or to a number of wavelengths by using an emitter capable of specific emission and/or using a detector capable of specific detection and/or by using filters interposed in an optical path between an emitter and a detector.

It has hereinbefore been disclosed that electron transport in the electron transport chain may be monitored for example by detecting the presence or absence of an electron in the biological component, or part thereof.

Thus, for example, if an absorption spectrum, or a part thereof, is used as a parameter to be detected, it will be understood that the spectrum or part thereof may differ depending upon whether, or not, electrons are passing along an electron transport chain.

Accordingly, by observing an absorption spectrum or part thereof the presence or absence of a substance capable of influencing electron transport in an electron transport chain may be detected.

Thus, by way of further example, the present invention further provides a method for detecting a substance, which method includes the step of subjecting a biological component, in which electron transport may be influenced by presence of a substance, to the influence of the substance, applying electro-magnetic energy, such as photo-energy, to the biological component and observing an absorption spectrum, or part thereof, of the biological component or part thereof.

It is to be understood that, by way of example, it may be possible to arrange for the presence of a substance to be detected to give rise to a reduction in observed intensity in an absorption spectrum or part thereof (e.g. by choosing to observe at a selected wavelength or wavelength region). However, it is further to be understood that it is possible alternatively, by way of example, to arrange for presence of the substance to give rise to an increase in observed intensity in an absorption spectrum or a part thereof (e.g. by choosing to observe at a selected wavelength or wavelength region); it is to be understood further that this increase in observed intensity may be achieved, for example, by choosing a wavelength or a wavelength region in which a peak occurs when the biological component or part thereof is in a state of electron transport activity which is reduced compared with normal. The term "normal" in the immediately preceding sentence means the situation which obtains when substance to be detected is not present and electron transport occurs as is usual for a given electron transport chain.

Where photo-energy (e.g. white light) is applied to a biological component in order to enable an absorption spectrum to be produced, and also where the biological component has a photosynthetic electron transport chain, it may be possible, if desired, to utilise the photo-energy both to produce the absorption spectrum and to "drive" the photosynthetic electron transfer chain.

In accordance with a further embodiment of the present invention a sensor may include a support material for the biological component.

Optionally, an emitter and/or a detector may be carried by a support material.

The support material may, for example, be a silicon wafer.

By way of further example, a support material may be subjected to micromachining to provide thereon or therein a region or area for accommodating a biological component.

It will be understood that micromachining may be carried out in any suitable manner such as those known for constructing structures on a small (e.g. micron) scale (e.g. photolithography).

Thus, in a further embodiment a support material may be constructed so as to be suitable for accommodating a whole cell or a plurality of whole cells.

For example, a suitable support material (e.g. a silicon wafer) may be provided with a groove into which a plurality of substantially rectangular microorganism cells may fit "end-to-end"; by providing an emitter at one end of the groove and a detector at the other end of the groove, energy passing through a plurality of cells (and hence encountering a plurality of electron transfer chains) may be monitored to enable the presence or absence of a substance to be detected.

By way of example, more than one groove with cells may be used; one of a plurality of grooves with cells may, optionally, be used to produce a reference signal.

According to a further aspect of the present invention there is provided a process for the preparation of a sensor, the sensor including a biological component, in which electron transport may be influenced by the presence of a substance to be detected, and optical means for detecting a parameter related to electron transport in the biological component or part thereof, which process includes the step of depositing a biological component on a support material.

Optionally, a biological component may be deposited on a support material and immobilised thereon.

Immobilisation may be carried out in any suitable manner, one example of which is growing of biological component into a defined region of a support material.

The present invention also provides a method for the detection of a substance which method includes using a sensor in accordance with the present invention.

It is to be understood that the term "parameter" as used in this Specification includes any property related to electron transport in a biological component or part thereof, which property is detectable by optical means.

CLAIMS

1. A sensor for detecting a substance which sensor includes a biological component, in which electron transport may be influenced by presence of substance to be detected, and optical means for detecting a parameter related to electron transport in the biological component or a part thereof.
2. A sensor as claimed in Claim 1 wherein the optical means includes an emitter and a detector.
3. A sensor as claimed in Claim 2 wherein the emitter is a laser, a light emitting diode or an optical fibre.
4. A sensor as claimed in Claim 2 wherein the detector is a photo-detector.
5. A sensor as claimed in any one of Claims 2, 3 or 4 wherein the sensor comprises a layer of cells, capable of providing a biological component, sandwiched between an optical emitter and an optical detector.
6. A sensor as claimed in any one of Claims 2, 3, 4 or 5 wherein the emitter and detector are arranged to co-operate so that together they form an optical means which may be specific to one wavelength or to a number of wavelengths.
7. A sensor as claimed in any one of Claims 1, 2, 3, 4, 5 or 6 wherein the sensor includes a support material for the biological component.
8. A sensor as claimed in Claim 7 wherein the support material is a silicon wafer.
9. A sensor as claimed in Claim 7 or Claim 8 wherein the support material is subjected to micromachining to provide thereon or therein a region or area for accommodating a biological component.
10. A sensor as claimed in any one of Claims 7, 8 or 9 wherein the support material is constructed so as to be suitable for accommodating a whole cell or a plurality of whole cells.
11. A sensor as claimed in any one of Claims 7, 8, 9 or 10 wherein a groove is supplied by the support material to accommodate a cell or a plurality of cells.

12. A sensor as claimed in any one Claims 1 to 11 wherein the biological component provides an electron transport chain comprising a photosynthetic electron transport chain or a mitochondrial electron transport chain.
13. A sensor as claimed in any one of claims 1,2,3,4,6 or 7 wherein the biological component comprises a single component of an electron transport chain.
14. A sensor as claimed in Claim 13 wherein the single component is immobilised.
15. A sensor as claimed in any one of Claims 1 to 14 wherein the sensor includes an entity which is capable of emitting energy upon receiving an electron from the biological component or part thereof thereby to enable emission from the entity to be detected by optical means to indicate the presence or absence of a substance to be detected.
16. A sensor as claimed in Claim 15 wherein the entity comprises a fluorescent redox-active species which is capable of accepting an electron from the biological component or part thereof and capable of exhibiting fluorescence in one of its redox states.
17. A sensor as claimed in any one of Claims 1 to 14 wherein the sensor includes a biological component which biological component or part thereof is such that it is capable of giving rise to fluorescence the fluorescence transient characteristics of which may be affected by the presence of a substance which influences electron transport in the biological component or part thereof, and means for detecting the fluorescence transient characteristics.
18. A method for detecting a substance, which method includes the steps of subjecting a biological component, in which electron transport may be influenced by presence of a substance to be detected, to the influence of the substance and detecting by optical means, which means includes an emitter and a detector, a parameter related to electron transport in the biological component or part thereof.

19. A method as claimed in Claim 18 wherein the biological component is a membrane within a cell.
20. A method as claimed in Claim 18 wherein the biological component is part of a cell membrane.
21. A method as claimed in any one of Claims 18, 19 or 20 wherein the biological component is provided by a whole cell.
22. A method as claimed in Claim 21 wherein the cell is a bacterium, an alga, a yeast or a plant cell.
23. A method as claimed in any one of Claims 18, 19, 20, 21 or 22 wherein the biological component provides an electron transport chain which comprises a photosynthetic electron transport chain or a mitochondrial electron transport chain.
24. A method as claimed in any one of Claims 18, 19, 20, 21 or 22 wherein the biological component provides an electron transport chain which derives electrons from catalysis of organic molecules.
25. A method as claimed in Claim 18 wherein the biological component comprises a single component of an electron transport chain.
26. A method as claimed in Claim 25 wherein the single component is immobilised.
27. A method as claimed in any one of Claims 18 to 26, wherein the substance to be detected is a toxic substance or a pollutant.
28. A method as claimed in any one of Claims 18 to 27, wherein the parameter is an absorption spectrum or a part thereof.
29. A method as claimed in any one of Claims 18 to 27, wherein an entity is used which entity is capable of emitting energy upon receiving an electron from the biological component or part thereof thereby to enable emission from the entity to be detected by optical means to indicate the presence or absence of a substance to be detected.
30. A method as claimed in Claim 29 wherein the entity comprises a fluorescent redox-active species which is capable of accepting an electron from the biological component or part thereof

and capable of exhibiting fluorescence in one of its redox states.

31. A method as claimed in any one of Claims 18 to 27 wherein the biological component or part thereof is such that it is capable of giving rise to fluorescence the fluorescence transient characteristics of which may be affected by the presence of a substance which influences electron transport in the biological component or part thereof and fluorescence transient characteristics are detected.

32. A method as claimed in Claim 28 which includes the step of subjecting a biological component in which electron transport may be influenced by presence of a substance to the influence of the substance, applying electro-magnetic energy to the biological component and observing an absorption spectrum, or part thereof, of the biological component or part thereof.

33. A method as claimed in any one of Claims 18 to 27 wherein photo energy is applied to a biological component or part thereof such that fluorescence is produced which fluorescence has two distinct fluorescence components comprising a fast transient component and a slow transient component.

34. A method as claimed in Claim 33 wherein the biological component comprises algae or photosynthetic bacteria, or chloroplasts, which contain photosynthetic reaction centres having photopigment complexes.

35. A process for the preparation of a sensor, the sensor including a biological component, in which electron transport may be influenced by the presence of a substance to be detected, and optical means for detecting a parameter related to electron transport in the biological component or part thereof, which process includes the step of depositing a biological component on a support material.

36. A process as claimed in Claim 35 wherein the biological component is deposited and immobilised on the support material.

37. A process as claimed in Claim 36 wherein immobilisation is effected by growing of biological component into a defined region of the support material.

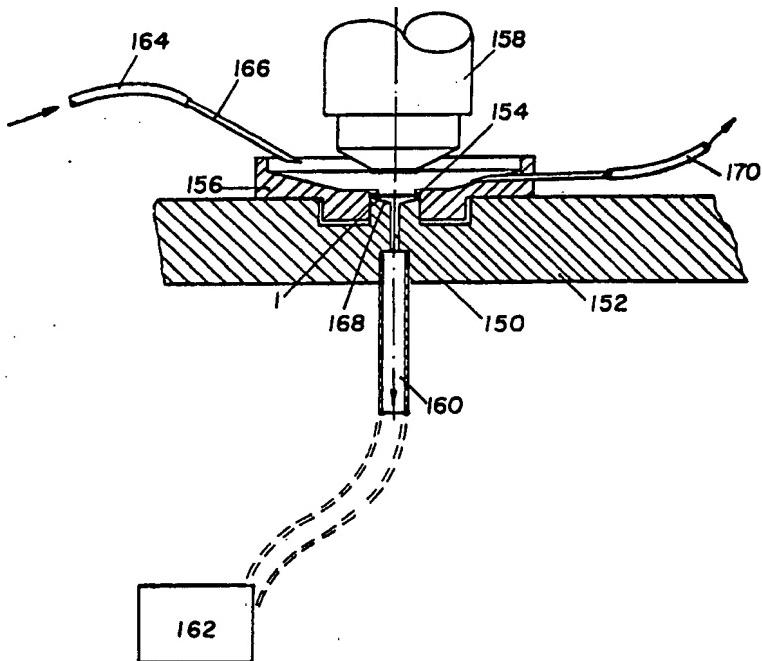
38. A method for the detection of a substance which method includes using a sensor as claimed in Claim 1.



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(54) Title: SYSTEM AND METHODS FOR CELL SELECTION



(57) Abstract

Individual living cells are placed at identifiable locations through the use of a carrier having a plurality of apertures which are 1) arranged in an ordered array, and 2) sized to hold individual cells. Once in the apertures, the cells can be studied, examined or manipulated on a one-by-one basis.

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SYSTEM AND METHODS FOR CELL SELECTION

5 The present invention relates to equipment and methods for cell selection and, more particularly, to equipment and methods for trapping individual cells at known locations. More generally, the invention relates to equipment and methods for studying, examining, or manipulating large groups of living cells, e.g., 10,000 or more individual cells, on a cell-by-cell basis.

10 In Swiss patent application Serial No. 2897/82-3, filed May 10, 1982, the relevant portions of which are incorporated herein by reference, we described equipment and methods for studying living cells on a cell-by-cell basis.

15 Briefly, our Swiss application described a process for placing individual living cells at identifiable locations comprising the steps of:

- 20 (a) providing a carrier having a plurality of apertures, the apertures being arranged in an ordered array and being sized to hold individual cells;
- (b) applying a fluid containing living cells to the carrier; and
- (c) applying a force to the cells to move the cells into the apertures.

25 Once in their individual apertures, the cells are studied, examined, and manipulated on a one-by-one basis. For example, the cells in the carrier apertures can be subjected to biological tests and particular properties of individual cells can be measured. As described in detail in our Swiss application, a particularly important application of this analysis approach involves using it to perform the Cercek SCM (Structuredness of Cytoplasmic Matrix) test for diagnosing cancer. See, for example, L. Cercek et al, Biophys. J., July 1978, Vol. 23, No. 1, p. 395 ff.

30 The present invention relates to new equipment and methods for practicing and using the methods and equipment described in our prior Swiss patent application. In particular, the objects of the present invention include providing improved methods and equipment for



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loading cells onto a carrier and preventing them from leaving a carrier; improved methods and equipment for exchanging the bathing fluid surrounding cells captured in the apertures of a carrier; improved methods and equipment for selecting and removing particular cells 5 from a carrier; improved methods and equipment for washing excess cells from a carrier; and improved carriers and methods for constructing such carriers.

To achieve the foregoing and other objects, the invention in accordance with certain of its aspects provides equipment and methods 10 for placing individual living cells at identifiable locations on a cell carrier wherein electromagnetic fields are used for loading the cells onto the carrier. In certain embodiments of these aspects of the invention, crossed electric and magnetic fields are used for loading, while in other embodiments, an electric field normal to the 15 surface of the carrier is used.

In accordance with other aspects of the invention, a time varying magnetic field is used to enhance the rate at which bathing fluid surrounding cells captured in the apertures of a carrier is exchanged. In addition to enhancing the rate of fluid exchange, such a 20 time varying magnetic field also has a massaging effect on the cells captured in the apertures.

In accordance with additional aspects of the invention, cells are held in the apertures of a carrier by adjusting the osmolarity of the bathing solution surrounding the cells so as to cause the cells 25 to swell.

In accordance with further aspects of the invention, electromagnetic fields are used to select and remove particular cells from a cell carrier. In certain embodiments of these aspects of the invention, a time varying electric field and a constant magnetic 30 field is used to select and remove cells having a particular charge to mass ratio. In other embodiments, a charged probe is used to remove individual cells from the carrier.

In accordance with additional aspects of the invention, equipment and methods are provided for washing excess cells from the surface of a cell carrier wherein a pressure differential is applied 35 across the cell carrier during the washing process. In certain preferred embodiments of these aspects of the invention, the carrier is



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washed by supplying fluid to its top surface through an inflow tube and removing it through a drain tube.

In accordance with still further aspects of the invention, improved cell carriers and methods for producing such carriers are provided. In particular, the invention provides coated cell carriers and cell carriers having apertures which include at least one vertical wall. The latter carriers are conveniently prepared using an ion bombardment process.

Further objects and features of the present invention will become more fully apparent from the following description of several embodiments of the invention based on the accompanying drawings, wherein:

Figs. 1A-1E are schematic illustrations, partly in sectional view, of preferred cell carriers of the invention.

Figs. 2-4 are scanning electron micrographs of copper carriers for use with the present invention.

Fig. 5 is a scanning electron micrograph showing a copper carrier coated with silicon.

Fig. 6 is a scanning electron micrograph showing a copper carrier having square-shaped apertures which are sized to hold and retain lymphocytes having a cross-sectional size of approximately 7 um.

Fig. 7 shows a typical experimental arrangement suitable for loading cells into carriers of the types shown in Figs. 2-4.

Fig. 8 is a scanning electron micrograph showing a carrier filled with lymphocytes.

Figs. 9-10 are scanning electron micrographs showing individual cells in individual apertures of a carrier.

Fig. 11 is a scanning electron micrograph showing the surface of the carrier prior to washing.

Fig. 12 shows the use of an electric field to drive cells into the apertures of a carrier.

Fig. 13 shows the use of crossed electric and magnet fields to drive cells into the apertures of a carrier.

Figs. 14-15 show the use of a time varying magnetic field to enhance fluid exchange about cells captured in a carrier.



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Fig. 16 shows the use of a time varying E field crossed with a constant B field to select a sub-population of cells captured in a carrier based on their charge to mass ratio.

As indicated above, the present invention relates to improved equipment and methods for studying, examining or manipulating living cells on a one-by-one basis wherein individual living cells are placed at identifiable locations on a cell carrier.

The cell carrier has an array of cell receiving holes, where for each hole, the location in the array, or address, is fixed and known. The holes extend from the carrier top side to a spaced apart bottom side. The holes have preselected configurations so that when a batch of cells passes over the carrier top side only preselected cells, based on their particular size, enter and become supported in the holes. Cells of sizes smaller than those of the selected cells pass through the holes, while much larger cells cannot enter the holes. Once the carrier is rinsed, only selected cells are located in its holes, one cell per hole at a fixed address.

Various cell carrier configurations are shown in Figs. 1A-1E. Carrier 1 includes base 3 in which are formed apertures or holes 2. The apertures or holes, as well as their arrangement, may have various configurations. In Fig. 1A, the holes are arranged in rows and columns along axes X and Y, respectively. As shown in Fig. 1B, the holes have larger openings at their tops than at their bottoms. The side walls of the apertures may converge continuously towards the opening at the bottom side 1b of the cell carrier, or in steps, as shown in Fig. 1C. Also, as shown in Fig. 1D, not all sides of the aperture need slope inwardly. Rather, a portion of the walls of the aperture can be essentially vertical so as to help capture and retain the cells in the apertures, especially when the cells are introduced into the carrier by being flowed across the top of the carrier in a direction substantially perpendicular to the apertures' vertical walls.

The shape of apertures 2 enables the cells to be effectively held to the carrier by applying means, such as a pressure difference between the upper and the bottom side of the carrier, or electromagnetic forces. Briefly, to first separate a particular group of cells from cells of other groups, since the cells in each



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group are of known size or sizes, which typically differ from those in other groups, the carrier 1 is chosen to have holes of sizes so that when the matter, e.g., blood, containing the various cell groups is placed on the carrier 1, effectively most if not all of the holes 5 are occupied by cells of the group of interest, one cell per hole.

For example, with regard to the SCM test referred to above, the holes are sized to be suited for receiving lymphocytes, among which there are two main sizes of about 7 um and about 10-15 um, the 7 um 10 lymphocytes being the cells of interest. To capture and retain this population of cells, it has been found that at the upper surface or side 1t of carrier 1, the apertures should have a cross-sectional dimension of approximately 10 um and that at the bottom surface or side 1b, they should have a cross-sectional dimension of approximate- 15 ly 5 um. In this way, the desired population of cells can easily enter the aperture without suffering substantial damage and yet, once in the aperture, the cells cannot pass out of the bottom of the car- rier.

In general the aperture should be shaped so that either at its 20 bottom side or at a cross-section intermediate sides 1t and 1b, the cross-sectional dimension is less than at the top side, so that a desired cell entering an aperture does not pass through the aperture, but rather is held therein. Fig. 1E illustrates an aperture config- 25 uration wherein the minimum cross-section is located in a plane inter- mediate between the top and bottom sides of the carrier. In addition to properly selecting the aperture's entering and exiting di- mensions, it is also important to choose the carrier thickness be- 30 tween the top and the level of the minimum cross-sectional dimension so that the size of the aperture is related to the size of the desired cells so that when a desired cell enters an aperture practi- cally the entire cell is within the aperture, thus preventing it from being washed out during washing of the carrier.

The carrier 1 is made of any appropriate matter, e.g., metals such as copper, gold, nickel, silver or others, or of plastic.

In addition to using pure metal or plastic carriers, in some 35 cases it is desirable to coat the carrier with various materials in order to change either or both of its chemical and mechanical surface characteristics. Examples of suitable coating materials include



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silicon, silicon dioxide and various inorganic glasses. When using such coating materials, or for that matter, when choosing a material from which to make an uncoated carrier, it is important to determine that the material does not interact with the cells in a way which will interfere with the test or tests to be performed.

For example, with regard to the SCM test referred to above, it has been found that a coating of SiO_2 on the carrier leads to activation of the cells (lymphocytes) which masks the response of these cells to stimulating agents. A similar activation is found with a mixture of silicon and Si_2O_3 . Pure silicon, on the other hand, does not lead to activation of the cells. Accordingly, for the SCM test, a carrier coating of silicon is appropriate, while a coating of silicon dioxide or silicon plus Si_2O_3 is not. Similar selections of coating materials can be readily made by persons skilled in the art for other types of diagnostic tests.

Scanning electron micrographs of a copper carrier for use with the present invention are shown in Figs. 2-4. Fig. 2 shows the top surface of the carrier at a magnification of 1000 X. At the level of this surface, the apertures have a cross-sectional dimension (diameter) of approximately 11 microns. The minimum cross-sectional dimension for these apertures is located in a plane intermediate the carrier's top and bottom surfaces and has a magnitude of approximately 4 microns. The spacing between this intermediate plane and the top surface of the carrier is approximately 6 microns. The spacing between apertures is approximately 15 microns. In general, the inter-aperture spacing should be kept as small as possible so as to maximize the chances that cells will come to rest inside apertures rather than on the portions of the carrier between apertures.

Figs. 3 and 4 show the bottom surface of the carrier of Fig. 2 at a magnification of 1000 X. Fig. 3 also shows a turned-up corner of the carrier. Examining the edges of the carriers of Fig. 2-4 reveals that the apertures have a vertical cross-sectional configuration of the type shown in Fig. 1E.

The carrier shown in Figs. 2-4 was prepared using a standard photo-etching technique of the type commercially employed to make transmission electron microscope grids. As is known in the art, that process, in its last stages, involves the deposition of metal on one



side of a preformed grid so as to increase the strength of the grid. When a transmission electron microscope grid is to be formed, the deposition step is carried on only for a short time so as to keep the size of the apertures as large as possible, i.e., to minimize the width of grid members which in turn minimizes the interference of the grid with the transmission of electrons through the specimen and to the electron detector. To form the carrier of Figs. 2-4, the deposition step, rather than being short, was continued for a relatively long period of time until enough metal was deposited on the back surface of the grid to fill in the apertures to the extent shown in the figures. As shown most clearly in Fig. 4, the deposited metal (copper) built up on the solid parts of the grid and overlapped into the apertures to close off the apertures and thus form the desired minimum cross-sectional dimension of the apertures.

Rather than using a deposition process to form carrier apertures of the desired configuration, other processes, in particular ion bombardment processes through masks of different thicknesses and the like, can be used. Such processes are particularly useful in preparing asymmetric apertures, such as the apertures shown in Fig. 1D.

Fig. 5 is a scanning electron micrograph at a magnification of 720 X illustrating a coated carrier. The base carrier in this case was formed from copper and the coating is pure silicon which was deposited on the carrier by vapor deposition. As can be seen in Fig. 5, coatings can be used to change (reduce) the cross-sectional dimensions of the apertures, as well as to provide an especially smooth and/or inert surface for contacting the cells.

Fig. 6 shows another uncoated, copper carrier, in this case having square rather than circular apertures. The cross-sectional dimension of the apertures at the top surface of this carrier is approximately 10 microns and the minimum cross-sectional dimension is approximately 5 microns. The minimum cross-sectional dimension lies in a plane approximately 7-8 microns below the top surface of the carrier. The spacing between apertures is approximately 12 microns.

The carriers of Figs. 2-4 and 6 are sized to be particularly well suited to capturing and retaining lymphocytes having a cross-sectional size of approximately 7 um. As will be evident to persons skilled in the art from the disclosure herein, other carriers



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having different aperture configurations can be constructed for capturing and retaining cells of different types and sizes.

As previously pointed out, the holes 2 in carrier 1 are regularly arranged over or in the carrier, e.g., in rows and columns, to enable a clear identification of the position of every hole 2, for example, by its X and Y coordinates in the plane of the carrier. In the described embodiment the holes are disposed in rows and columns, extending perpendicularly to each other, thereby forming a matrix-like structure. The number of holes is chosen depending on the number of cells to be carried. For example, with 100 holes per row and column there is a total of 10,000 holes to carry 10,000 cells on the carrier of the described embodiment, each with its unique position in X and Y.

To practice the method of the present invention, a few drops of the solution containing the cells, e.g., blood containing the lymphocytes, are dripped onto the cell carrier. A force, for example, a pressure differential, is applied across the carrier to move the cells into the apertures. The liquid passes through the holes in the carrier. However, the cells remain on the carrier. Since the sizes of the holes 2 are chosen to accommodate lymphocytes only, they enter the holes. Each hole accommodates only one cell. Excessive and other cells may be washed off the surface of the carrier, such as cells of sizes so great that they can't enter any hole, and/or excess cells more than the number of holes. Thereafter, in order to prevent the cells in the holes from leaving the carrier, they may be fixed thereto by various means, e.g., by applying a continuous pressure differential across the holes, by changing the osmolarity of the bathing solution to cause the cells to swell, by covering the carrier by an adhesive, colloidable matter, and by electrically charging the carrier, as well as by external electric and/or magnetic fields.

Fig. 7 illustrates a typical experimental arrangement which has been used to load cells into carriers of the types shown in Figs. 2-6.

Carrier 1 is held in place above orifice 150 in plate 152 by means of collar 154 of solution basin 156. The collar presses the carrier against the portion of plate 152 which surrounds orifice 150 and creates a seal between that portion and the carrier. This seal



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prevents substantial numbers of cells from passing around the edges of the carrier, rather than being captured in the apertures.

Orifice 150 is connected by outflow tube 160 to pump 162. The pump serves to produce a pressure differential across carrier 1 which 5 pulls the cells into the apertures in the carrier. It has been found that a more uniform filling of carrier 1 can be achieved by providing a shallow taper 168 at the mouth of orifice 150. This taper reduces the amount of time required to fill the apertures at the perimeter of the carrier.

10 Basin 156 is configured so as to allow microscope objective 158 to be brought close enough to carrier 1 so that the apertures in the carrier can be brought into focus. Solutions are provided to basin 156 by one or more inflow tubes 164 which are conveniently connected to syringe needles 166. The inflow tubes are used to introduce various 15 bathing and reagent solutions to basin 156. The inflow tubes are also used to wash excess cells off the top surface of carrier 1. In this case, fluid is removed from basin 156 by means of drain tube 170. Cells are applied to carrier 1 using a standard syringe. During 20 this operation microscope objective 158 and basin 156 are moved apart to allow ready access to carrier 1. The level of fluid in basin 156 is monitored during the testing of cells and, as necessary, fluid is added to the basin to keep the cells captured in carrier 1 continuously submerged in liquid.

A typical procedure used to capture and retain human lymphocytes 25 in a carrier using the apparatus of Fig. 7 was as follows. First, a sample of human whole blood was obtained in the standard way. A plasma fraction of this blood was then obtained by either centrifuging the sample at approximately 100 g for approximately 6 minutes or by incubating the sample at 37°C for approximately a half an hour. In 30 either case, the plasma fraction had a pinkish cast indicating the presence of red blood cells. The red blood cell/white blood cell ratio of the plasma fractions used to fill carriers was estimated to be approximately 30/1.

Once obtained, the plasma fractions were diluted with phosphate 35 buffered saline until a cell concentration of either approximately 6×10^6 cells/cc or 12×10^6 cells/cc was reached. So that the cells would fluoresce and thus be easily seen under the microscope during



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loading onto the carrier, fluorescein diacetate (FDA) was added to the cell suspension at a concentration of approximately 2.5 uM and the cells were incubated with FDA for approximately 10-30 seconds prior to being applied to the carrier.

5 To make sure that the carrier was free from contamination, phosphate buffered saline was added to basin 154 with carrier 1 in place and pumped through carrier 1 by means of pump 162. Pump 162 was adjusted to produce a pressure differential across carrier 1 in the range of 0.5-5.0 cm of water.

10 The cells were applied to carrier 1 by bringing a standard syringe containing the cell suspension into the vicinity of the carrier. For the 6×10^6 cells/cc concentration it was found that three drops of the cell suspension applied near to, but not directly on, the carrier were adequate to essentially fill all of the apertures in
15 a carrier having approximately 7500 holes, while for the 12×10^6 cells/cc concentration level and the same size carrier, one drop applied directly to the carrier was found to be sufficient. In either case, essentially complete filling of the carrier occurred within a period of seconds to minutes, depending on how well collar 154 sealed
20 the carrier to plate 152.

Fig. 8 is a scanning electron micrograph at a magnifications of 1000 X showing the carrier filled with lymphocytes. The fixation process used to prepare this micrograph causes the cells to contract. This makes them appear somewhat smaller than the apertures. When the
25 cells were alive, they essentially filled the whole aperture with their tops at or just below the top surface of the carrier.

Figs. 9-10 are scanning electron micrographs at a magnification of 6600X showing individual cells in individual apertures. The cell shown in Figs. 9 is a lymphocyte, while the cell in the aperture in
30 Fig. 10 is an erythrocyte. Because erythrocytes are smaller than lymphocytes and are relatively flexible, if pressure had continued to be applied across the carrier, the erythrocyte shown in Fig. 10 would have passed down and out of the aperture.

Fig. 11 is a scanning electron micrograph at a magnification of 480X showing the carrier surface prior to washing to remove excess cells and debris. For the apparatus of Fig. 7, washing is done using inflow tube 164 and drain tube 170. Note that the pressure



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differential created by pump 162, as well as the configuration of the apertures, serves to hold the cells in their apertures during the washing process.

As can be seen in Fig. 11, prior to washing there are individual lymphocytes in individual apertures, but the top of the carrier is covered with both excess lymphocytes and erythrocytes, as well as other cell types and debris. Comparing Fig. 11 with Fig. 8, which shows the carrier surface after washing, clearly demonstrates the effectiveness of the washing process in removing excess cells and debris.

In the examples described above, a pressure difference across carrier 1 has been used to drive the cells into the carrier apertures and then retain the cells in the apertures. Other forces can also be used for these purposes.

For example, Fig. 12 shows the use of an electric field to drive the cells against the carrier and into the apertures. The field is oriented perpendicular to the top surface of the carrier. As is known in the art, biological cells, including lymphocytes, normally carry a net electrical charge, or, by adjusting the pH or other parameters, can be made to have a net charge. The electric field shown in Fig. 12 will accordingly cause cells, e.g., positively charged cells, to move towards the carrier and into the apertures, as desired. Of course, if it is negatively charged cells which one wants to capture on the carrier, one only needs to reverse the direction of the electric field.

The use of an electric field as the driving force can lead to electrolysis problems with uncoated metallic carriers. One solution to this problem is to coat the carrier with a non-conductor, as described above. Another solution, illustrated in Fig. 12, is to give the carrier a shape which localizes most of the electrolysis effects at points distant from the apertures where the cells are captured. In particular, in Fig. 12, the carrier is provided with ears or projections 172 which concentrate the electric field and thus the ionic current and electrolysis effects in regions away from the main body of the carrier. Such ears will also attract cells, but in general there will be an abundant excess of cells so that even if there is some concentration of cells in the regions of the ears, there will



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still be enough cells near the body of the carrier to fill the apertures.

As an alternative to using an electric field oriented perpendicular to the surface of the carrier, crossed electric and magnetic fields parallel to the surface of the carrier can be used to drive the cells into the apertures. As shown in Fig. 13, in this case, the electric field causes the charged cells to move across the top of the carrier, while the magnetic field produces a $v \times B$ force towards the surface of the carrier for positively charged cells. Again, negatively charged cells can be selected by reversing the direction of the B field. The use of a B field to drive the cells into the apertures has the advantage that once the cell comes to rest in the aperture, the force on the cell due to the driving force ceases because v is now equal to zero. In contrast, a pressure differential driving force continues to exert a force on the cells even after they have been captured in apertures, although in general this force is too small to cause damage to the cells.

In addition to using E and B fields to apply cells to the carrier, these fields can be used to enhance the rate of fluid exchange around individual cells and to select specific cells captured on the carrier based on such parameters as the cell's charge to mass ratio.

With regard to fluid exchange, Fig. 14 shows the use of a time varying magnetic field normal to the surface of the carrier to cause cells to rotate about their axes inside apertures. More specifically, the time varying magnet field generates a circular or tangential electric field parallel to the plane of the carrier. The magnitude and direction of such a field is described by Maxwell-Faraday law, also known as Lenz's law. This field acts on the fixed charges on the surface of the cell membrane and thus causes the cells to rotate about an axis parallel to the magnetic field. It should be noted that once the cells begin to rotate their cell membranes will experience either an inward or outward squeezing force resulting from the $v \times B$ (Lorentz) interaction between the charges on the membrane and the applied B field (see Fig. 15). Whether the force is inward or outward will depend on the sign of the cell's surface charge and the orientation of the B field. In essence, the time varying magnetic field, in addition to rotating the cells, will also have a massaging



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effect on them. Furthermore, there will be a tendency for the rotating cell, which in effect is a magnetic dipole, to move parallel to the magnetic field. In addition to the effects on the cells, the field also interacts with the charged ions in the bathing medium 5 causing them to move in circular paths.

With regard to selecting particular types of cells from among the population captured on the carrier, Fig. 16 shows an arrangement for selecting those cells having a particular charge to mass ratio. As shown in that figure, a time varying, e.g., sinusoidal, electric 10 field is applied across the carrier and a constant magnetic field is applied parallel to the top surface of the carrier. The response of individual cells to the electric field will depend on the frequency of the field and the cell's charge to mass ratio. Accordingly, by varying the frequency of the electric field, specific subgroups of 15 cells can be made to move sufficiently far out of their apertures so that the force due to the magnetic field acting on the moving cell will cause it to move in the plane of the surface of the carrier. By means of surface washing during this process, these selected cells can be removed.

20 In addition to the foregoing, electric fields can be used to select individual cells. For example, individual cells can be removed from the carrier by a local electric field created by bringing a charged probe into the vicinity of a particular cell's aperture. Groups of cells can be similarly removed from the carrier and 25 moved to a desired location by using a movable array of probes, where selected probes in the array can be charged to a value sufficient to attract and move a cell from its aperture.

Although particular embodiments of the invention have been described and illustrated herein, it is recognized that modifications 30 and variations may readily occur to those skilled in the art, and consequently, it is intended that the claims be interpreted to cover such modifications and equivalents.



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What is claimed is:

1. A method for placing individual living cells at identifiable locations comprising the steps of:

5 (a) providing a carrier having a plurality of apertures, the apertures being arranged in an ordered array and being sized to hold individual cells;

(b) applying a fluid containing living cells to the carrier; and

10 (c) applying an electromagnetic force to the cells to move the cells into the apertures.

2. The method of Claim 1 wherein the electromagnetic force is produced by an electric field oriented perpendicular to the surface of the carrier.

15 3. The method of Claim 1 wherein the electromagnetic force is produced by crossed electric and magnetic fields oriented parallel to the surface of the carrier.

4. Apparatus for placing individual living cells at identifiable locations comprising:

20 (a) a carrier having a plurality of apertures, the apertures being arranged in an ordered array and being sized to hold individual cells; and

(b) means for applying an electromagnetic force to the cells to move the cells into the apertures.

25 5. The apparatus of Claim 4 wherein the means for applying an electromagnetic force includes means for producing an electric field oriented perpendicular to the surface of the carrier.

6. The apparatus of Claim 5 wherein the carrier is made of metal and is coated with a non-conductor.

30 7. The apparatus of Claim 5 wherein the carrier is made of metal and has a configuration which localizes electrolysis effects at points distant from the locations of the apertures.

8. The apparatus of Claim 7 wherein the carrier includes projections which extend away from the portion of the carrier which includes the apertures.

35 9. The apparatus of Claim 4 wherein the means for applying an electromagnetic force includes means for producing crossed



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electric and magnetic fields oriented parallel to the top surface of the carrier.

10. In a method for exchanging the bathing fluid surrounding cells captured in the apertures of a carrier, the improvement comprising applying a time varying magnetic field normal to the surface of the carrier to cause the cells to rotate about their axes inside the apertures.

11. Apparatus for exchanging the bathing fluid surrounding cells captured in the apertures of a carrier comprising:

10 (a) means for applying and removing bathing fluid from the carrier; and

(b) means for producing a time varying magnetic field normal to the surface of the carrier to cause the cells to rotate about their axes inside the apertures.

15 12. In a method for holding cells in the apertures of a carrier, the improvement comprising washing the carrier to remove excess cells and debris, and then adjusting the osmolarity of the bathing solution surrounding the cells so as to cause the cells to swell.

20 13. A method for selecting cells which have a particular charge to mass ratio from among a population of cells captured in the apertures of a carrier comprising the steps of applying a time varying electric field across the carrier and applying a constant magnetic field parallel to the surface of the carrier to move cells having the particular charge to mass ratio out of their apertures.

25 14. The method of Claim 13 including the additional step of washing the surface of the carrier while said electric and magnetic fields are being applied.

30 15. Apparatus for selecting cells which have a particular charge to mass ratio from among a population of cells captured in the apertures of a carrier comprising first and second means for respectively producing electric and magnetic fields, said electric field being time varying and being directed across the carrier, said magnetic field being constant and being directed parallel to the surface of the carrier, said electric field causing cells which have the particular charge to mass ratio to move sufficiently far out of their



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apertures so that said magnetic field can move the cells in the plane of the surface of the carrier.

16. The apparatus of Claim 15 further comprising means for washing the surface of the carrier.

5 17. A method for removing an individual cell from an aperture formed in a carrier comprising the steps of charging a probe and subjecting the cell to the local electric field produced by the charged probe to move the cell out of its aperture.

10 18. The method of Claim 17 wherein a group of individual cells are removed from their apertures by charging an array of probes and subjecting the cells to the local electric fields produced by the array to move the cells out of their apertures.

15 19. Apparatus for removing a cell from an aperture formed in a carrier comprising a probe and means for charging the probe to a value sufficient to attract and move the cell from its aperture.

20 20. Apparatus for removing a group of cells from the apertures of a carrier comprising an array of probes and means for charging selected probes in the array to a value sufficient to attract and move a cell from its aperture.

20 21. In a method for washing excess cells and debris from a carrier which has a plurality of apertures which are filed with cells, the improvement comprising applying a pressure differential across the carrier to hold the cells in the apertures during the washing process.

25 22. The method of Claim 21 wherein the washing is performed by supplying fluid to the top surface of the carrier through an inflow tube and removing it through a drain tube.

30 23. A carrier for holding individual cells at identifiable locations comprising a base made of a first material and having a plurality of apertures therethrough, said apertures being arranged in an ordered array and being sized to hold individual cells, and a coating made of a second material and covering at least a portion of said base.

35 24. The carrier of Claim 23 wherein the base is a conductor and the coating is a non-conductor.

25. The carrier of Claim 24 wherein the base is metal and the coating is an inorganic glass.



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26. The carrier of Claim 24 wherein the base is metal and the coating is Si, SiO₂, or a mixture of Si and Si₂O₃.

27. The carrier of Claim 24 wherein the base is metal and the coating is Si.

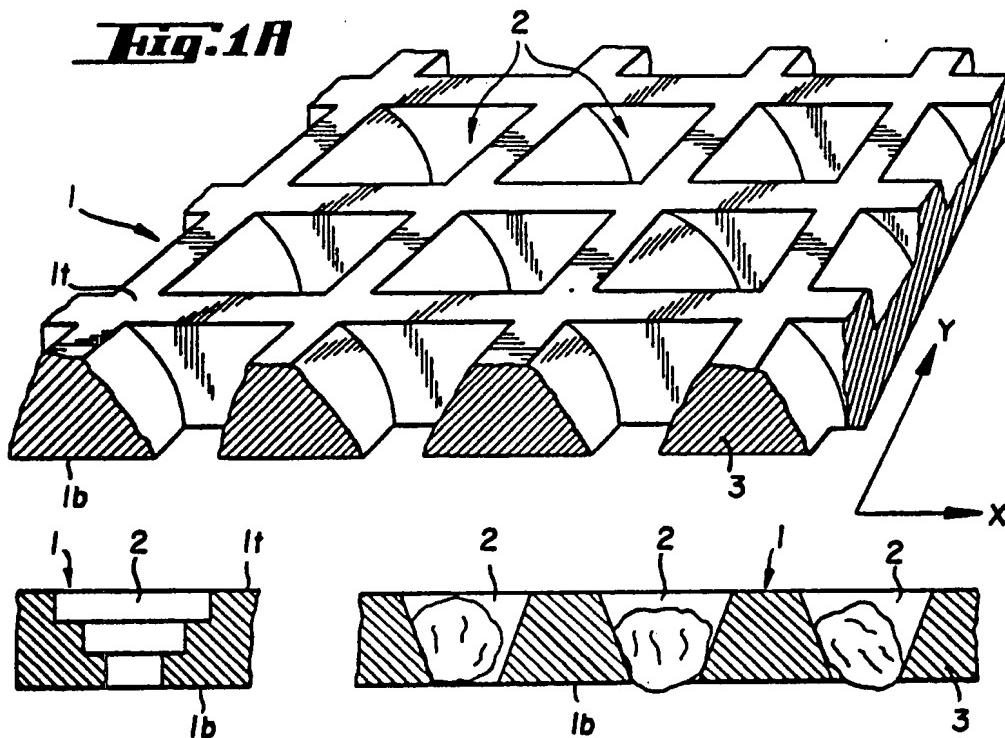
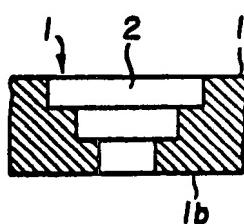
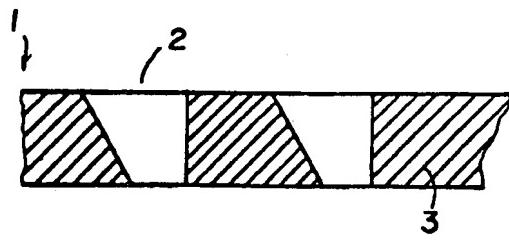
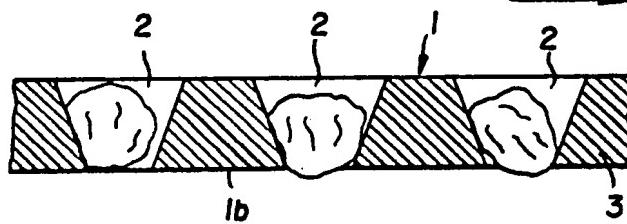
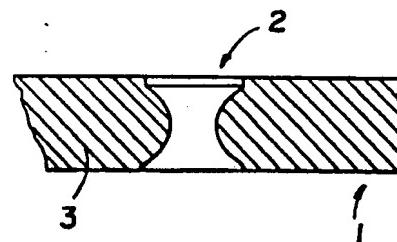
5 28. A method for forming a carrier for holding individual cells at identifiable locations comprising creating apertures in a base by subjecting the base to ion bombardment through masks of different thicknesses, the apertures being in an ordered array and being sized to hold individual cells.

10 29. A carrier for holding individual cells at identifiable locations comprising a base having substantially parallel top and bottom surfaces and having a plurality of apertures therethrough, said apertures (a) being arranged in an ordered array, (b) being sized to hold individual cells, and (c) having side walls which extend between said top and bottom surfaces of said base, said side walls including a first portion which converges inward towards the center of the aperture and a second portion which is substantially perpendicular to said top and bottom surfaces of said base.

15 30. The carrier of Claim 29 wherein the apertures are formed by ion bombardment.



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Fig. 1A**Fig. 1C****Fig. 1B****Fig. 1D****Fig. 1E**

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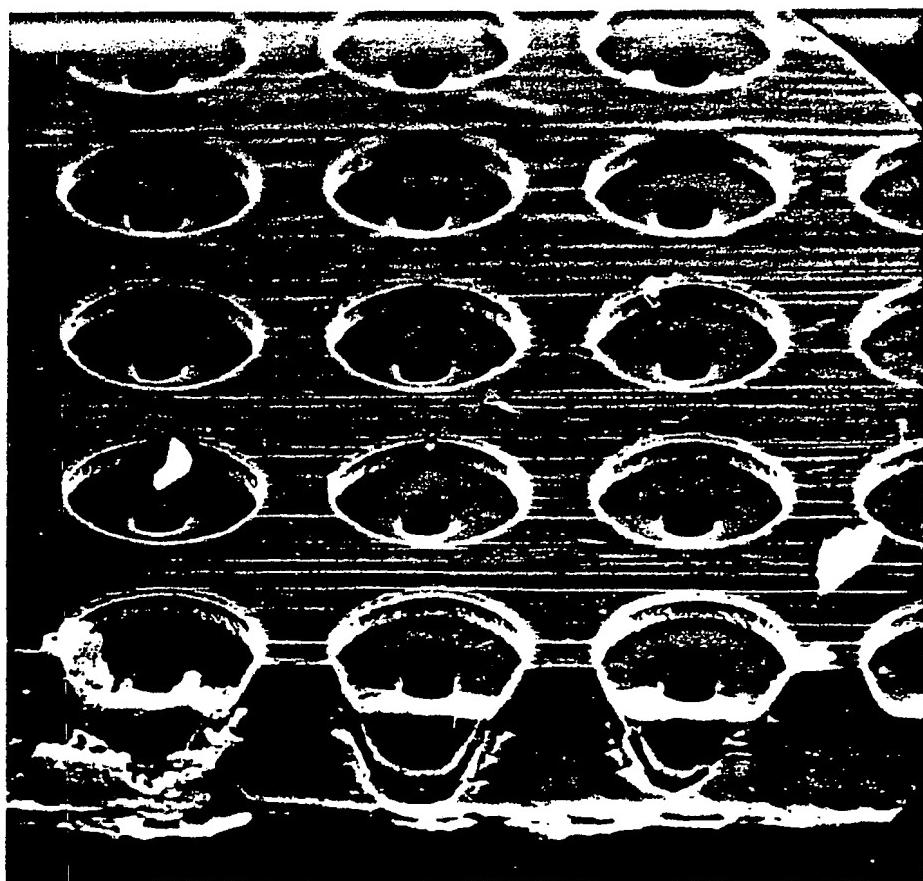


Fig. 2

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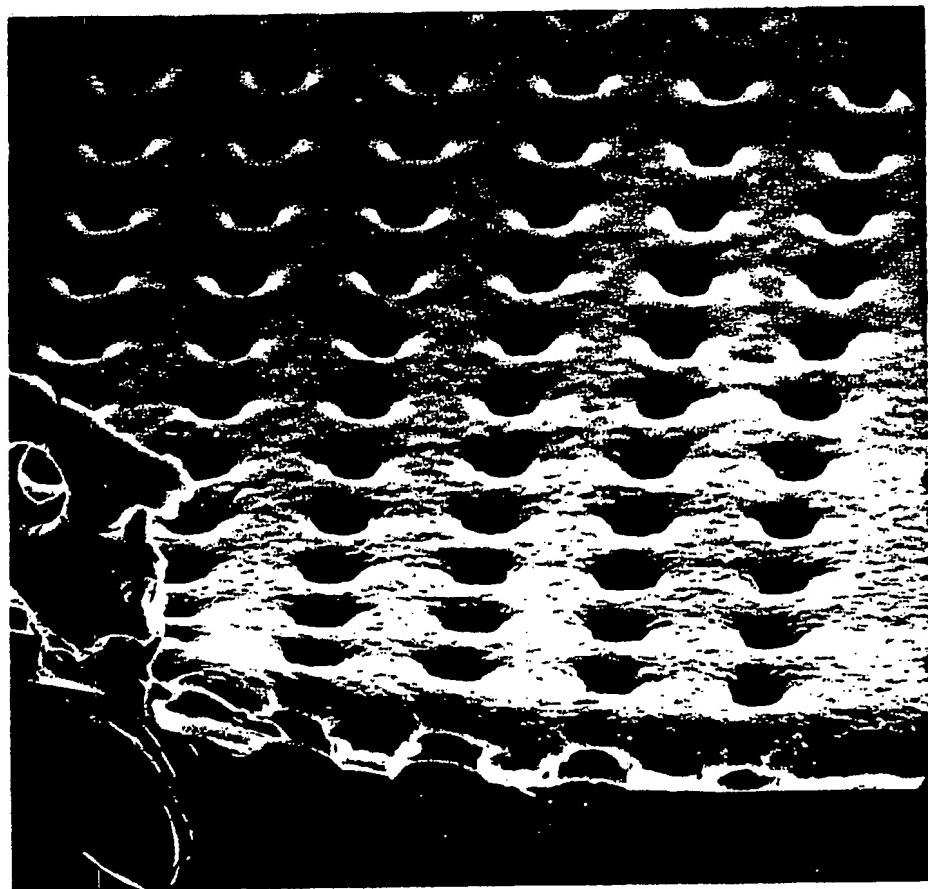


Fig. 3

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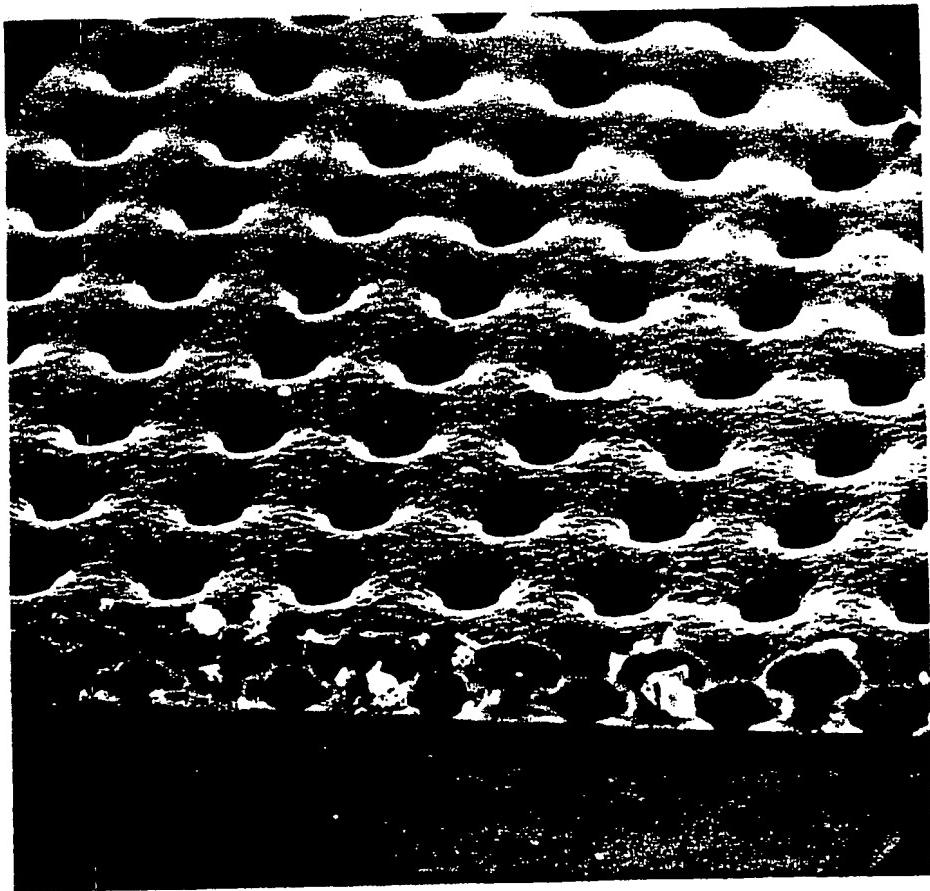


Fig.4

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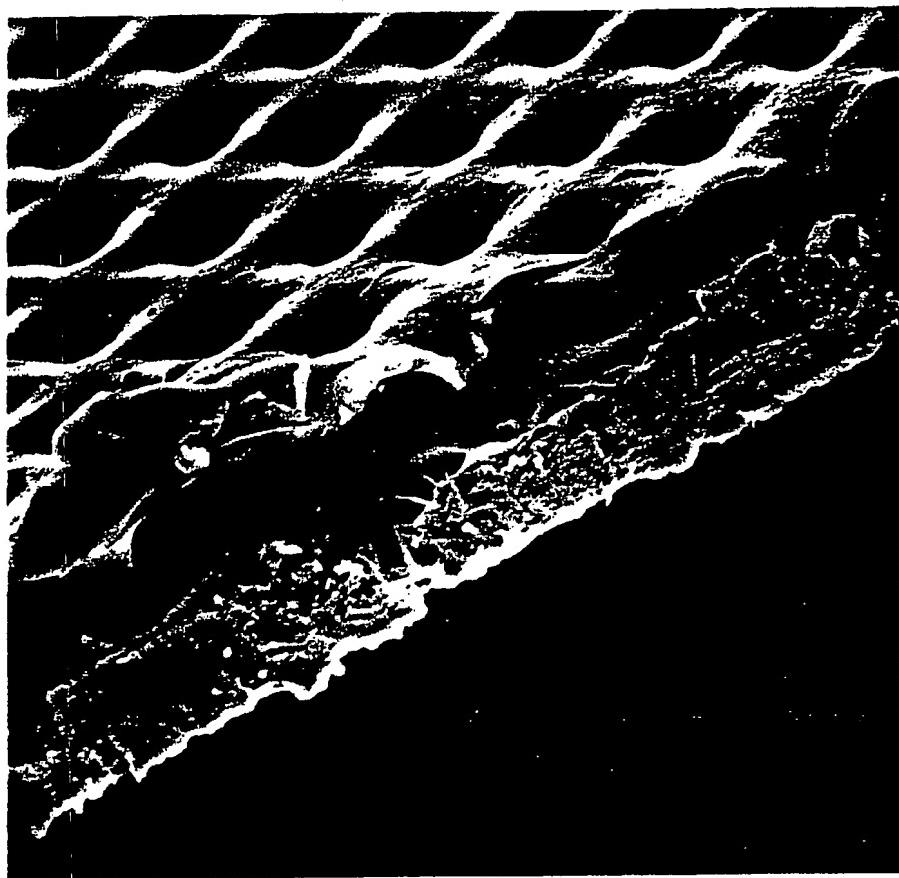


Fig. 5

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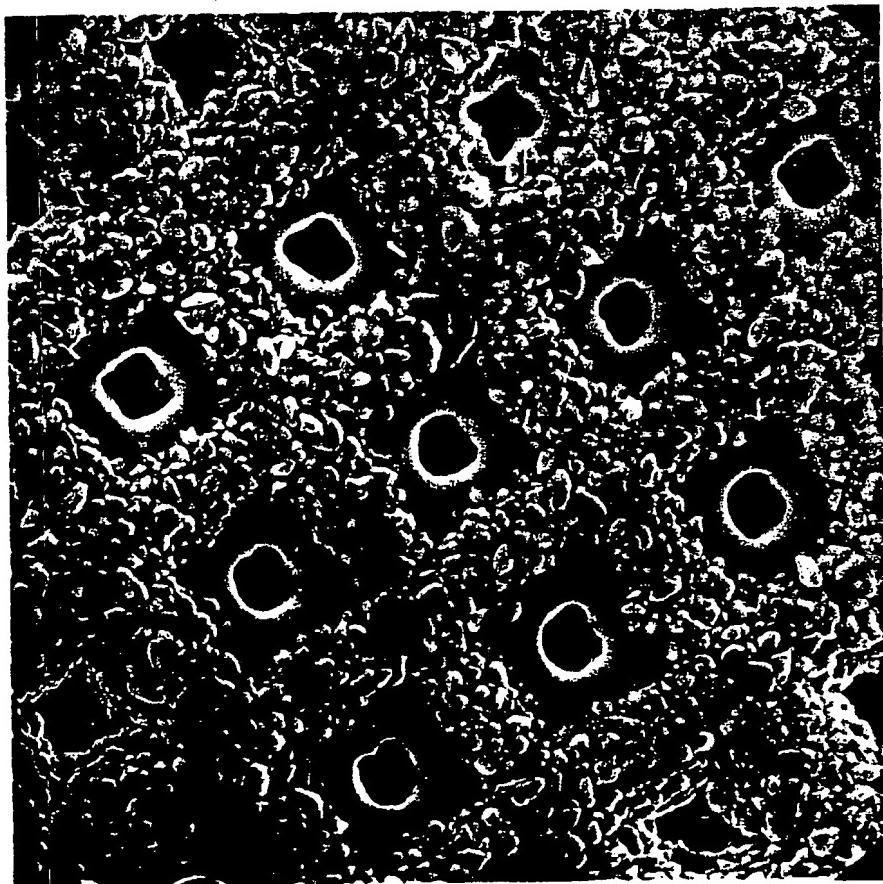
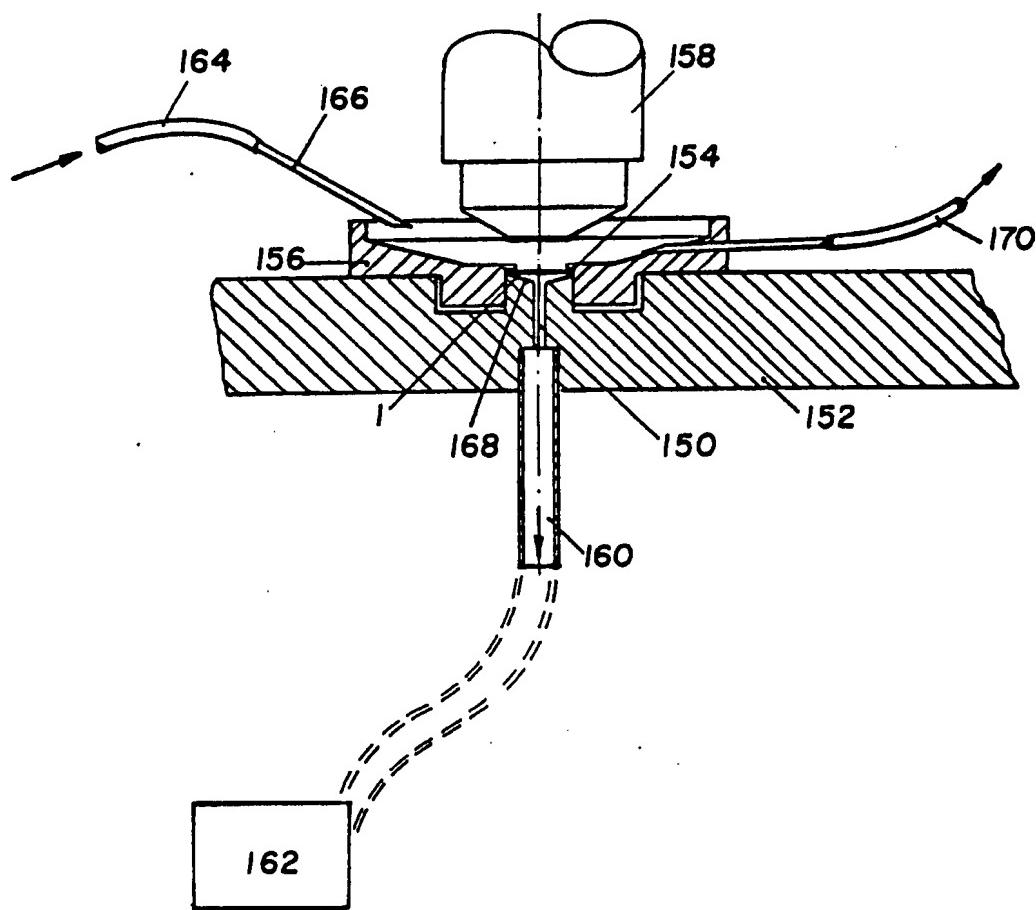


Fig. 6

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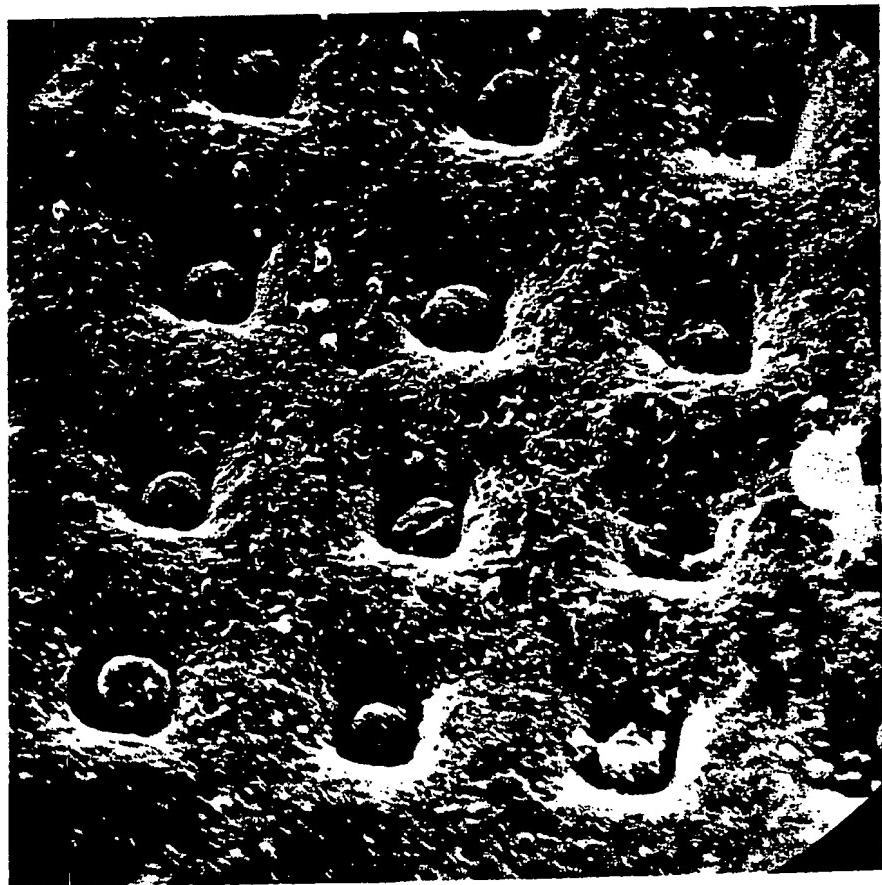


Fig. 8

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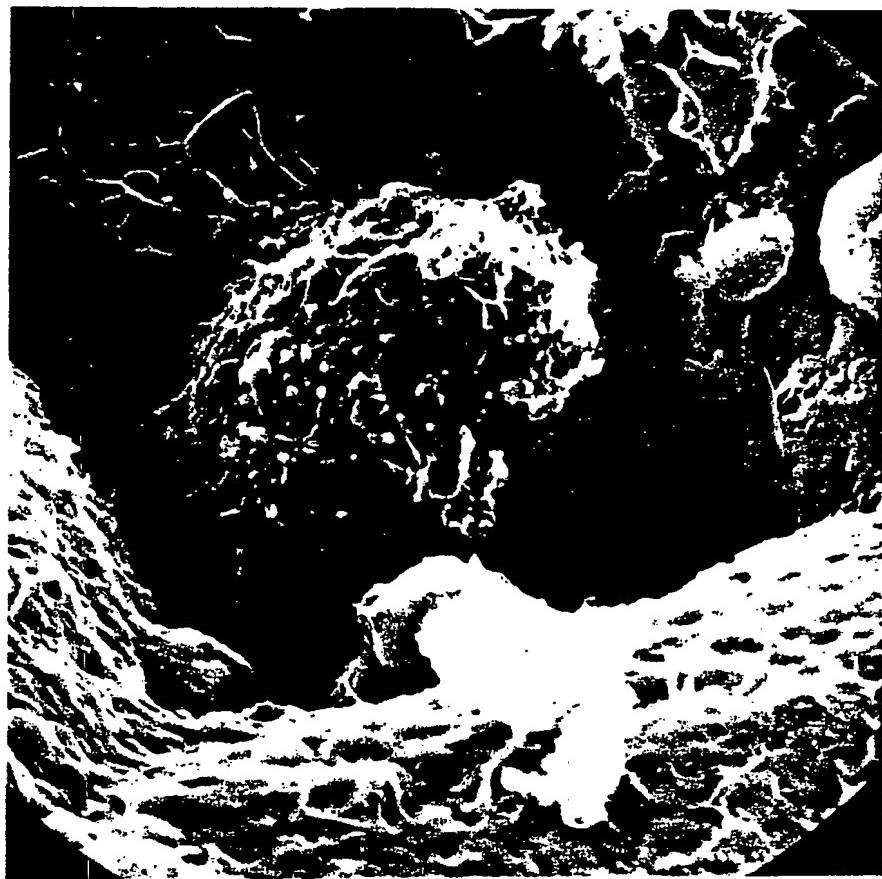


Fig. 9

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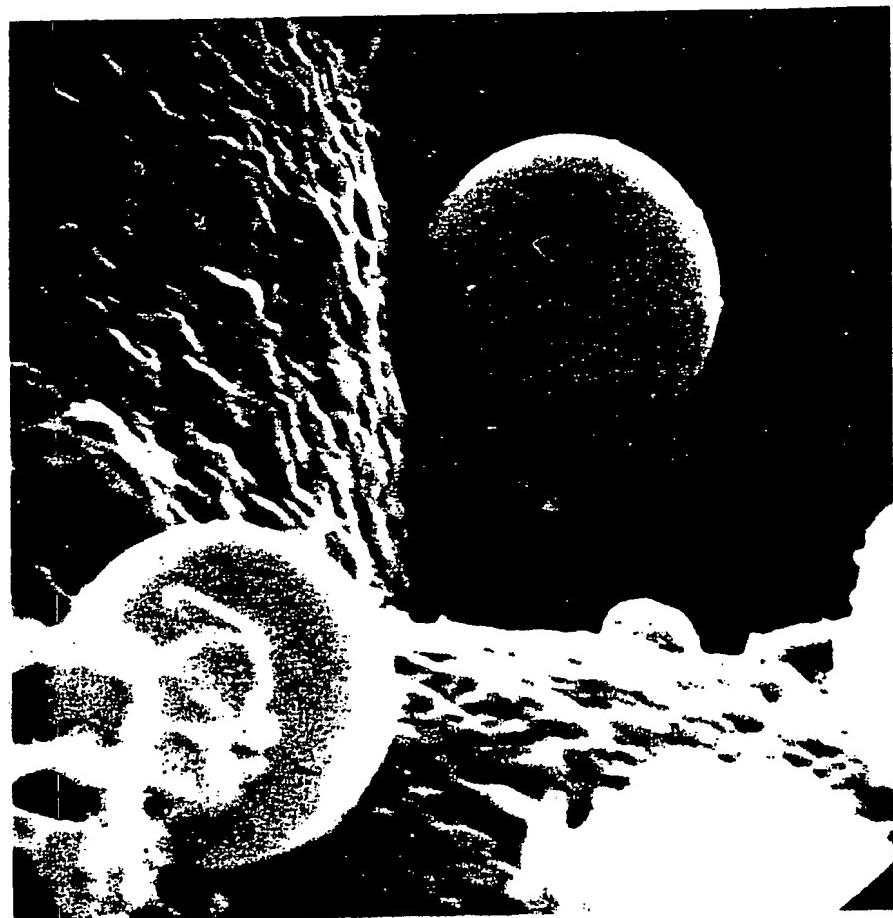


Fig. 10

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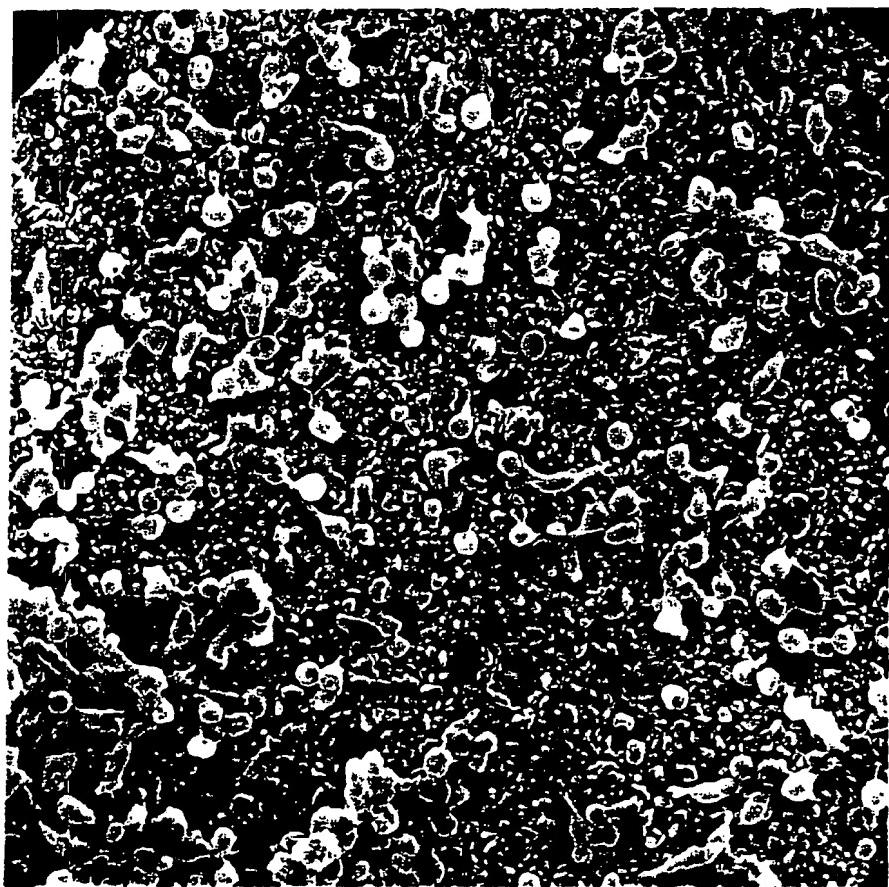


Fig.11

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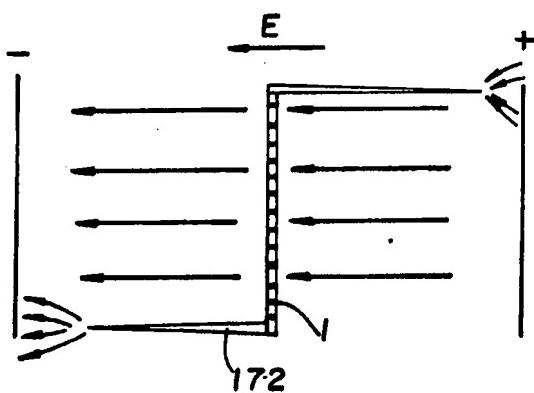


Fig.12

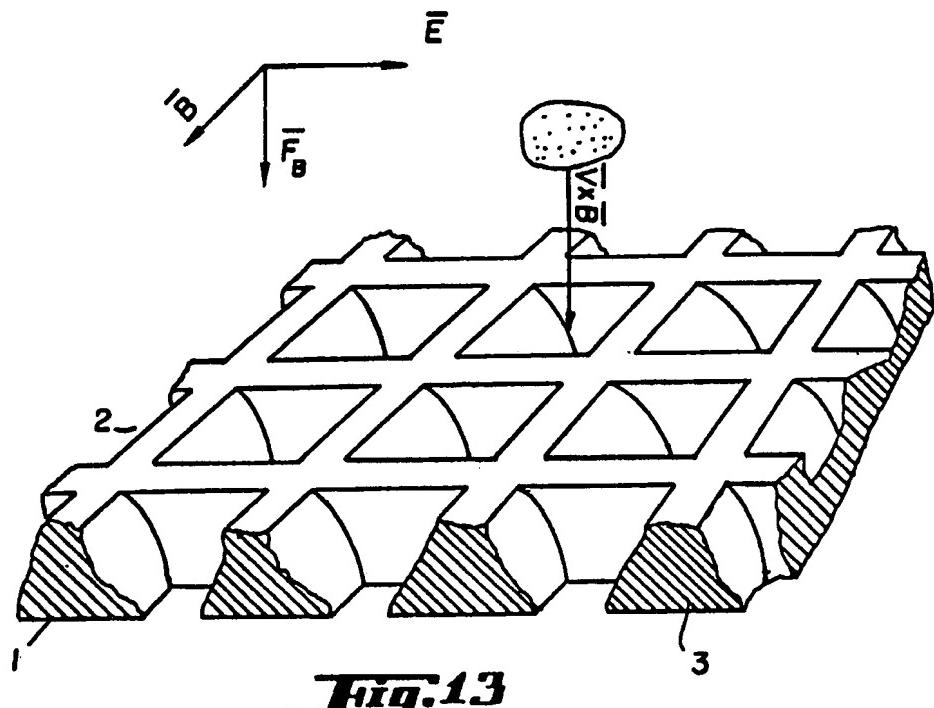
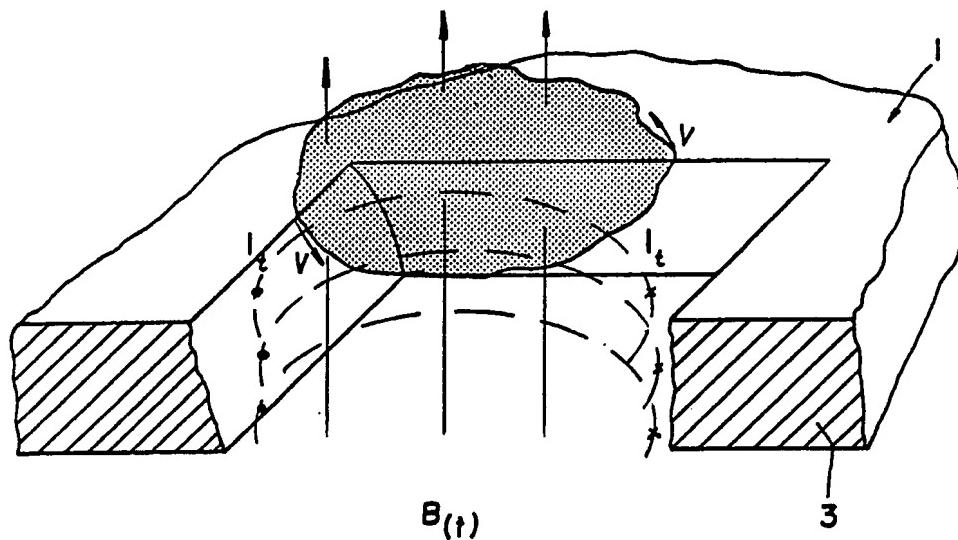
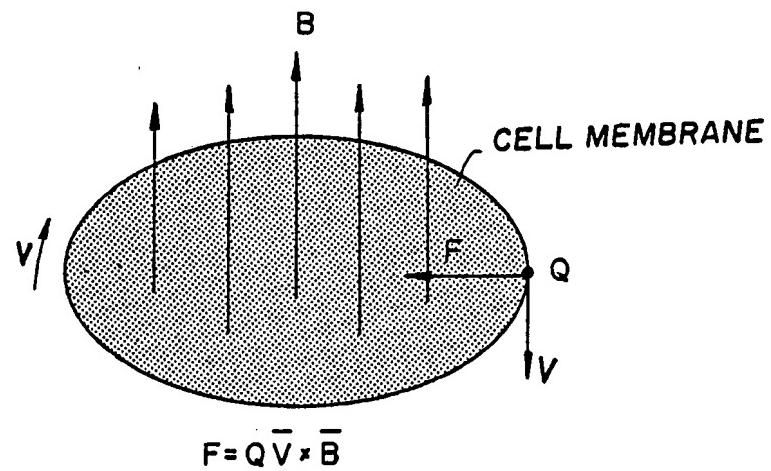


Fig.13

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**Fig.14****Fig.15****SUBSTITUTE SHEET**

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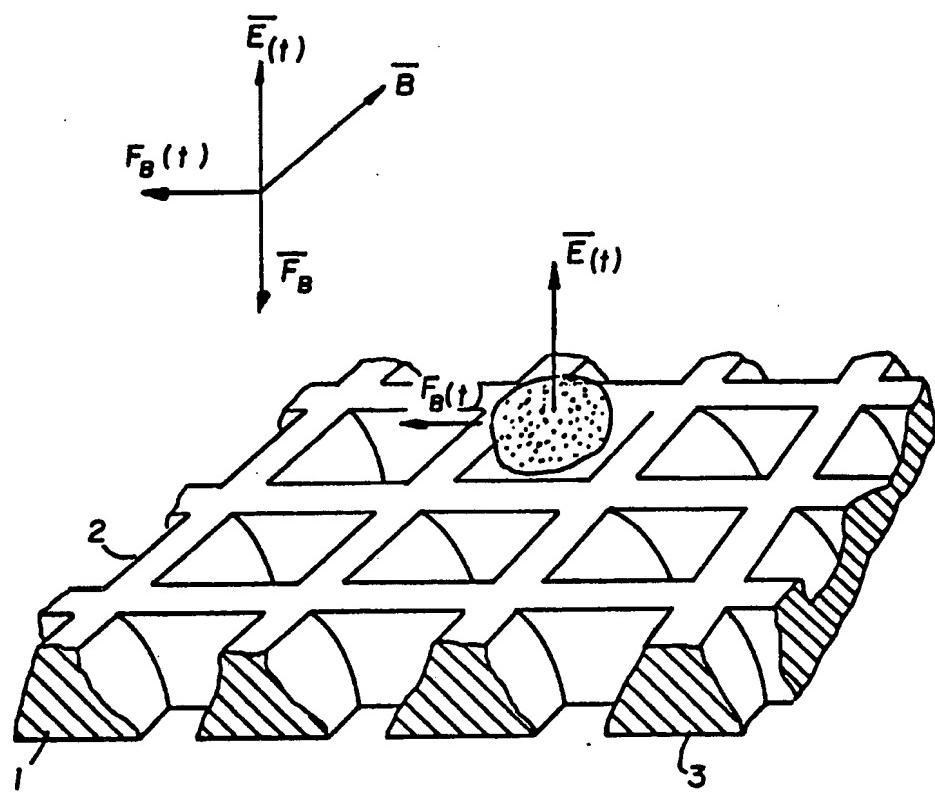


Fig. 16

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/01829

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC
U.S. CL. 435/30,173,261,287,291,311,803; 422/101; 436/63,177,178
 INT. CL. C12Q 1/24; C12N 13/00,1/02; C12M 1/00,1/34,1/12;

Cont.
next p

II. FIELDS SEARCHED

Minimum Documentation Searched *

| Classification System | Classification Symbols |
|-----------------------|--|
| U.S. | 435/30,173,261,287,291,311,803; 422/101; 436/63,177 210/222,695; 209/38,243; 204/180R, 186,301,305 178 |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Computer Search: Biological Abstracts and Chemical Abstracts Data Bases

III. DOCUMENTS CONSIDERED TO BE RELEVANT **

| Category * | Citation of Document, ** with indication, where appropriate, of the relevant passages *** | Relevant to Claim No. *** |
|------------|---|---------------------------|
| Y | US,A, 4,055,799 (Coster et al) 25 October 1977 | 1-9,23-27,29, 30 |
| A | N, Laboratory Equipment Digest, Volume 18, number 10, Issued October 1980, High-Voltage FFE Separates Cells Without Damage. | 1-9, 13-16 |
| Y | US,A, 3,874,851 (Wilkins et al) 1 April 1975 | 21, 22 |
| A | US,A, 3,874,851 (Wilkins et al) 1 April 1975 | 1-9,23-27,29, 30 |
| A | US,A, 4,162,850 (Warren) 31 July 1979 | 1-9,23-27,29 30 |
| A | US,A, 4,025,306 (Studer) 24 May 1977 | 1-9,23-27,29, 30 |
| Y | US,A, 2,910,406 (Novak) 27 October 1959 | 23-27, 29, 30 |

* Special categories of cited documents: **

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search *

31 January 1985

Date of Mailing of this International Search Report *

07 FEB 1985

International Searching Authority *

ISA/US

Signature of Authorized Officer *

(Randall E. Deck)

~~EST/US~~ 84 / 01829

I. CLASSIFICATION OF SUBJECT MATTER:

US. 210/222, 695; 209/38, 243; 204/180R, 186, 301, 302, 305
INT. B01L 11/00; B01D 35/06, 57/02, 13/02; B03C 1/30, 1/02,
5/02

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|--------|
| A | US,A, 2,910,406 (Novak) 27 October 1959 | 1-9 |
| Y | US,A, 2,923,669 (Poitr.s) 2 February 1960 | 21, 22 |
| A | US,A, 1,915,568 (Gortner et al) 27 June 1933 | 1-9 |
| A | US,A, 3,368,963 (Hall) 13 February 1968 | 1-9 |
| A | US,A, 4,089,765 (Dudley) 16 May 1978 | 1-9 |

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹⁰

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:
 1. Claim numbers because they relate to subject matter¹¹ not required to be searched by this Authority, namely:

2. Claim numbers, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹², specifically:

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING¹³

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category* | Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No ¹⁸ |
|-----------|--|------------------------------------|
| A | US,A, 3,190,827 (Kok et al) 22 June 1965 | 1-9 |
| Y | US,A, 3,207,684 (Dotts, Jr.) 21 September 1965 | 3,9,13,15 |
| A | US,A, 4,374,644 (Armstrong) 22 February 1983 | 12 |
| A | US,A, 3,929,583 (Sharpe et al) 30 December 1975 | 1,23-27,29,30 |
| A | US,A, 4,052,163 (Patzner) 4 October 1977 | 23-27,29,30 |
| A,E | US,A, 4,441,972 (Pohl) 10 April 1984 | 1-9 |
| A | US,A, 3,719,583 (Ustick) 6 March 1973 | 1-9 |
| A | US,A, 4,326,934 (Pohl) 27 April 1982 | 1-9 |

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